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MECHANISMS OF BIOLOGICAL EFFECTS OF PENETRATING RADIATIONS

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To THE biophysicist, the study of the biological effects of penetrating radiation is a particularly interesting field because of the intimate connection of the radiation effect with the problem of growth. I wish to mention a few of the unique properties of radiations:

First, some radiations can penetrate the cell wall and interact with internal components of cells. The cell wall provides protection against many chemical agents.

Secondly, radiation seems to inhibit the mechanisms controlling growth before it seriously interferes with other cell functions. Radiation thus becomes a suitable tool for study of cell division.

Thirdly, the amount of energy which the cell must absorb if cell division is to be inhibited is extremely small compared to the energy turnover of the cell as a machine, or compared to the amount of energy needed from other sources to cause lethal effect. For example, many cells are inactivated by an amount of penetrating radiation which, as heat, would be only sufficient to raise cell temperature by a few thousandths of a degree. In contrast, thermal death due to raised temperature would take 10,000 times as much absorbed energy as radiation death. In fact, for some cells it is known, since the pioneer work of Dessauer (1, 2), that production of a single ion pair can cause lethal effect in the cell. In these cases, at least, lethal effect must be due to a chain of reactions.

Fourthly, as is generally known, a single interaction with ionizing radiation is able to make an inheritable change, or mutation, in the gene complement of cells. Radiation induced mutations are usually unfavorable; occasionally they can make cell metabolism more efficient. Thus radiation may have an important role in both destruction and evolution of life.

In order to comprehend at least a few of the complex phenomena involved in radiation effects on animals, it is useful to look at the explanation of the effects on unicellular organisms, and in order to understand these, one should study the physicochemical effects of radiation in inert chemical systems. The field is so large that all I can hope to give you is a list of some of the recent experimental approaches, then to describe, in a little more detail, some experimental findings of special interest to me. Excellent review articles by Bacq and by Curtis are available.

INTERACTION OF RADIATION WITH MATTER

Penetrating radiations either produce ionization directly in their passage through matter, as beta rays, alpha rays and protons; or they produce ionizing secondary particles as X-rays, gamma rays or neutrons. In the process of ionization, a negative electron is knocked out of an atom, leaving it positively charged. The negative electron attaches itself to a molecule, so that within 10^{-14} seconds a positive-negative ion pair is formed. The ions of the ion pair in liquid media often dissociate or interact with neutral molecules and form radicals or more stable chemical compounds. In addition, a number of atoms also

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become 'excited' due to the passage of an ionizing ray, that is, one of the orbital electrons absorbs energy and is elevated to an excited state. The molecule in which the excited atom is located then might dissociate, emit a light quantum, interact with another molecule, or degrade the excitation energy by collisions with other molecules into vibrational and rotational energy. Dose may be defined, for the purposes of this discussion, as the energy absorbed in form of ionization and excitation per gram tissue. The radiochemical and radiobiological action of the ionizing radiation depends not only on dose, but on the geometrical distribution of ion pairs along each track. The number of ion pairs along a unit length of track is the 'specific ionization.' Among radiations available to experimentation, the specific ionization varies over a range of 100,000.

DIRECT ACTION

As to the mode of biological action of radiations, many early studies indicated that direct interaction of the ions with biological material occurs in some instances, causing splitting or fragmentation of large biological molecules. Classical example of such a process is the splitting of hemocyanin molecules as demonstrated by Svedberg and Brohult (3).

Experimentally, breakdown of macromolecules can be accomplished with different radiations: neutrons from the chain reacting pile, x-rays, electrons, deuterons and alpha particles from the cyclotron; it can be performed on dry, biologically active molecules in the crystalline state, or in liquid suspensions. Some intriguing results to date include the following findings:

a) Breakdown of macromolecules by radiation sometimes occurs in fragments of well definable molecular weight (4).

b) In addition to breakdown, association of macromolecules can occur (5).

c) Quantitative study of the action of cyclotron-produced protons on crystalline enzymes leads to a novel method for determination of molecular weight (6).

d) When insulin was subjected to intense radiation with neutrons, a large part of the biological activity was retained in molecular fragments which had a variety of molecular weights (7).

The above instances indicate the potentialities of the 'molecular fragmentation' method in the breakdown study of biologically important molecules.

INDIRECT ACTION

In the second type of interaction of radiation with living matter, the primary ionization or excitation occurs at some distance from the molecules affected. As first clearly demonstrated by Dale (8), the indirect action occurs when the short-lived ions disappear, and give rise to radicals and intermediate chemical compounds in the liquid medium. The intermediates have much longer lifetimes than the ions themselves; and they can, in turn, chemically interact with protein molecules. In very dilute aqueous suspensions of enzyme molecules, the primary radiation effect is on water; and most of the effect is due to water decomposition products.

The decomposition of pure water, in its main features, is quite well understood, due to the work of Lind (9), Fricke (10), Weiss (11) and others (12, 13). We know that ionized water molecules almost instantly decompose into H and OH radicals, that these can readily interact; in the absence of oxygen, some hydrogen gas and hydrogen peroxide is formed. When oxygen is also present, dissolved in water, the radical HO_2 forms, which in turn enhances hydrogen peroxide formation. In the presence of organic matter, the formation of H_2O_2 is not observed; but the evolution of hydrogen is greater. When the water contains sulfhydryl enzymes with active SH groups, these are oxidized to form S-S bonds, presumably via interaction with the radicals.

Because the yield, lifetime, and interactions of intermediates depend on the chemical composition of the cell medium, one expects large variation of radiosensitivity due to environmental factors, such as temperature, pH, and composition of the medium. In contrast, the 'direct' effect is independent of these factors. Indeed, in living cells, striking environmental influences of radiosensitivity have been demonstrated.

Radiation sensitivity of an enzyme system depends on the composition of the aqueous medium When substances are added to the medium that can compete for the radicals and other intermediates produced by radiation, due to the competition, the enzyme system can be partially protected from inactivation. An example, given by Barron *et al.* (14), is the protection from x-rays of phosphoglyceraldehyde dehydrogenase with added glutathione. The same protective phenomenon was demonstrated in mammals by the brilliant work of Harvey Patt (15)

and his associates, who have recently shown that the amino acid cysteine, if administered to animals shortly before irradiation, increases their tolerance, so that twice the x-ray dosage is needed for lethal effect. Similar results have been achieved by Belgian workers with cyanide (16). At this meeting, workers from the Oakridge National Laboratory (17) presented data on an impressive array of organic substances which protect bacteria.

Indirect biological effects also depend on specific ionization (18). Spacing of ion pairs influences the yield of intermediates. In oxygen free water, for example, the dense ionization of alpha rays produces a high yield of H_2O_2 molecules. The yield of H_2O_2 remains small when sparsely ionizing x-rays are used. The enzyme phosphoglyceraldehyde dehydrogenase is partially protected from α-rays by catalase (19). Many biological systems are more affected by dense rather than by sparse ionization.

If some of the actual biological effects are caused by intermediate chemical compounds and not directly by ionization processes themselves, it should be feasible to produce similar effects with applied chemical compounds. Several types of radiation effects, notably chromosome breaks, mutations and carcinogenic effects (20, 21), have indeed been reproduced by a variety of chemicals. Typical agents that have similar effect to radiation are nitrogen mustards and peroxides.

Substances that contribute to formation of intermediates after exposure can increase the destructiveness of ionizing radiation. Thoday and Read (22) have shown that oxygen dissolved in the cell medium is known to increase radiation sensitivity; Lacassagne (23) demonstrated that baby rats are more resistant under anaerobic conditions than in the presence of oxygen; and Dowdy *et al.* (24) showed that anoxic rats survive higher doses of radiation than normal ones.

Barron recognized the possibility that if radiation effects are due to chemical reactions, they should be, at least in part, chemically reversible. By the addition of glutathione he was able to reactivate some sulfhydryl enzymes previously inactivated by radiation. A similar approach might possibly be of import in future therapy of radiation effects.

In the process of ionization or excitation, certain molecules receive a good deal more energy than is necessary for an ordinary chemical reaction. The new field of radiation chemistry concerns itself with such unusual reactions. It is expected that ionization causes a number of irreversible reactions which would otherwise only occur at high temperatures and pressures.

Recently Garrison *et al.* (25) have shown that carbon dioxide, dissolved in water and irradiated by alpha particles, produces some organic molecules, notably formic acid. Other investigators have for some time been studying a number of other interesting reactions. Mund (26) has shown polymerization of simple carbon compounds with occasional formation of cyclic molecules from straight chain substances. Dainton (27) has shown that certain low molecular weight monomers (e.g., acrylonitrile) in aqueous solution become polymerized into long chain polymers.

MECHANISM OF EFFECTS OF RADIATIONS AT THE CELLULAR LEVEL

In view of the great complexity of radiochemical reactions, many of which involve higher order reactions, it is surprising that the relationship of dose to certain types of biological effects is often relatively simple.

In many cases the dose-effect relationship has the properties of a monomolecular reaction. There are, however, cases which deviate from this simple relationship. The lethal effect in unicellular organisms may occur in the irradiated cells themselves, e.g., they fail to divide, and after the lapse of some time, they might undergo lysis. In another form of the lethal effect, the formation of colonies is inhibited, but the irradiated cell lives on. Although it was known for some years that irradiation of the cell nucleus brings about lethal effect more effectively than irradiation of the cytoplasm (28), it seems likely that inhibition of cell division is due to genetic damage only in some species of cells and to extragenic destruction in others.

In association with Professor R. E. Zirkle of the University of Chicago, the Donner Laboratory group 2 years ago initiated a study of the mechanisms involved in the inhibition of yeast cells, *Saccharomyces cerevisiae*. French workers, Laterjet and Euphrussi (29), as well as Beam (30), have independently worked on the same problem and our respective results are not in disagreement. The approach was to test the relationship of dose to inhibition of cell division in haploid cells and compare the results of data obtained after irradiation of vegetative diploid colonies.

The morphological nutritional requirements

and growth rate of the haploid and diploid colonies were closely similar, and it was assumed that any differences in response to radiation effect would be due to the presence of paired genes and chromosomes in the diploid against the presence of one set in the haploid cells. It was further reasoned that if the radiation effect is most important in extrogenic and extranuclear components of the cells, there should be no great difference in the radiation sensitivity of haploid and diploid cells. Detailed results of this investigation will be published elsewhere (31). We may, however, note here some of the conclusions. One great difference was found in the radiation sensitivity and in the dose affecting the relationship between haploid and diploid cells, the latter being about five times as resistant as the former. The relative shapes of the survival curves of cells in which cell division was not inhibited were compared, and the conclusions were mathematically in fair agreement with the following concepts:

a) Cell division is controlled in haploid cells from a number of mutually independent 'sites' (loci sensitive to radiation). Inactivation of any of these sites brings about inhibition of cell division. Inhibition can be the result of interactions initiated from a single ionization event. The probability of inhibition is proportional to dose, and the survival curve is a simple exponential function of dose.

b) In diploid cells, cell division is controlled from a number of mutually independent pairs of 'sites' which have similar functions to the sites in haploid cells. To inhibit cell division, it is necessary to inactivate both sites of an essential pair (allele). At least two ionization events are needed to accomplish this. The probability of inhibition of cell division is not proportional to the dose. The survival curve is of a sigmoid type.

c) Comparison of haploid and diploid survival curves can be made from the relative shape of the curves and the assumption that each 'site' is inactivated with the same probability. One concludes that in the haploid cells, there are about 16 sites essential to cell division; and in the diploid, 16 pairs of such sites.

Biochemical synthesis in cells is known to be under genetic control (32). It seems plausible to associate the sites sensitive to irradiation with independent biochemical processes controlled by genes.

The number of radiation sensitive sites is only an approximation which is expected to vary with environmental factors. In a medium very rich in various nutrients, there may be alternatives for each biochemical pathway so that the number of sensitive sites might be different. Yet it is of interest to note that this number is much smaller than the estimated total number of genes, and no proof is available as yet showing that single genes are affected. A mechanical disturbance, such as a chromosome break with loss of fragments during attempted cell division might be an acceptable concept of the mechanism involved.

From the above considerations, two important conclusions follow. In the first place, one would expect cells of higher ploidy of the same strain to show higher radiation resistance. The second consideration follows from the statistical nature of the distribution of radiation induced defects. If a colony of diploid cells is irradiated by a single dose, a number of the cells in which cell division is not inhibited will carry impaired genetic defects corresponding to inactivation of impaired radiation sensitive sites. Such defects should be hereditary and would make the cells less efficient in aspects of growth and metabolism, and more sensitive to radiation than normal diploid cells with no defects. Indeed, this was found to be the case experimentally. In certain experiments, as much as 50 per cent of the progeny of preirradiated diploid yeast cells showed an inheritable increase in radiosensitivity, accompanied in some cases by reduced vigor of growth and metabolism. Haploid cells surviving a preirradiation do not show these changes, which is a strong argument in favor of the recessive nature of such radiation induced effects.

The implication of these findings lies close at hand. By studying the biochemical nature of the abnormalities in the recessively defective diploid cells, one should get valuable data, not only concerning the components of radiation effects on cells, but also relating to essential steps in cell division. Since the general types of defects are limited to about 16 kinds (and combinations of these), it may be possible to proceed systematically. The further pursuit of this kind of study may also be of interest since it might uncover some clues concerning the nature of interaction between allelic gene pairs.

The experiments on yeast cells, carried out under a variety of environmental conditions, give clear indication that the mechanism in-

olved is indirect: the ion pairs do not need to e produced at the 'site' of their action.

The probability that an ion pair, through some ntermediate process, can find an essential genetic omponent of the cell, is rather small; hence, many other ion pairs and interactions are produced elsewhere within the cell before the nhibition of cell division occurs. These, collectively, may be responsible in the yeast cells for liverse effects of radiation, delay in cell division, swelling, etc. In many species of cells they may even be responsible for the actual lethal effect.

When a large colony of diploid cells is irradiated, even with a dose much larger than the 50 per cent LD, one finds some cells which have received no genetic defect at all. This is due to the statistical spatial distribution of ionization. Such cells may play an important role in the 'recovery' phenomenon well known in radiobiology. When many generations of growth have passed between one irradiation and the next, most of the cells present in the preirradiated colony exhibit normal radiation resistance. This may be due to the fact that diploid cells left unaffected by radiation, and those which mutated back to normal, outgrow the ones with genetic defects.

It would be desirable to have detailed information on the mechanism of radiation effects on other microorganisms. Much work is being done in this field; unfortunately we do not as yet have sufficient information to indicate whether or not the process of inhibition of cell division is similar in each case.

RADIATION EFFECTS ON MAMMALS

There is a very long step between physiological simplicity of yeast cells and the complexity of mammals. Many experiments will have to be performed before the radiation effects are understood in the latter. However, there are certain similarities I wish to point out.

When a part of the body of a young animal is irradiated with a sublethal dose, one often finds a prolonged retardation of growth of that tissue lasting for several generations of cells. For example, an irradiated limb in a baby animal may remain stunted for a long period of time. Though there are many factors involved, e.g. radiation effects on vascular bed and connective tissue, some of the surviving irradiated cells have possibly unpaired genetic defects which slow the rate of growth and metabolic efficiency of each cell. Histologically, regeneration in such tissues often follows a curious pattern. In the middle of a seemingly devastated area of impaired cells, a small island of normal young cells appears. These cells appear to originate from a single cell, possibly one that did not suffer any genetic damage from the irradiation. This phenomenon is observed in therapeutically irradiated tumor tissue as well. One might speculate on the frequency and importance of impaired genetic defects produced by radiation in the diploid germ cells of mammals. By reduction division these produce haploid gametes carrying lethal genetic defects, which in turn would give rise to recessive lethals in the offspring (32). The presence of such processes will have to be proven experimentally.

Radiation effects on the tissues of higher animals should not be directly compared to those of microorganisms. The functioning of each organ in the body depends on the functioning of other organs. When the total body is irradiated, one obtains not only direct radiation effects on the cells of each tissue, but indirect effects as well, due to changes in internal environment. The internal environment is chiefly modified by humoral agents. Investigations carried out with partial body irradiation of small animals have shown the validity of some fairly general rules:

a) There are important, remote effects on various organs when they are themselves shielded from radiation, but other parts of the body are irradiated. These effects probably originate from deficiencies in the blood composition or toxic substances carried by the blood. According to Hevesy (33), nucleic acid synthesis is diminished if a remote part of the body is irradiated. Kelly and Jones (34) amplified this finding by showing that selective irradiation of the liver with a radioactive colloid materially affects nucleic acid synthesis in tumors. Van Dyke and Huff (35) irradiated one member of a pair of parabiotic rats. Epilation of the hair extended not only to the irradiated animal, but also to its shielded twin. They have thus demonstrated that a toxic substance or a depletion of some essential blood constituent has transmitted the effect via the cross-circulation between the animals.

b) Animals survive a greater dose of partial body irradiation than of whole body irradiation. The data of Bond *et al.* (36) show that partial irradiation of the abdomen increases the LD 50 in rats by a factor of 2. Similar recent informa-

tion was gained by Jacobson *et al.* (37, 38) who have shown that shielding of the spleen alone increases the 50 per cent LD by a factor of 2 and that shielding of almost any part of the body (e.g. head) affords some protection against radiation effect. When one member of parabiotic twins is irradiated, the lethal dose for that member is very significantly increased (35). All these results strengthen the assertion that in mammals, a significant fraction of acute radiation effect must be extragenic and mediated by the humoral agents.

Selective irradiation of a portion of the animal body is not easy to perform with x-rays. The Donner Laboratory group (39) is utilizing high energy deuteron ion beams which allow irradiation of a small deep-seated region of the body without materially affecting the overlying tissues. In one of the experiments, done in collaboration with the Institute of Experimental Biology (40), the anterior pituitary gland of rats was irradiated, and a study of the overall and differential effects on secretion of pituitary hormones is under way.

A few years ago attempts for therapy of the acute radiation syndrome seemed to be quite hopeless. With the realization that many of the symptoms are due to humoral agents, we c now look forward with some confidence to t development of at least partial therapeutic mea ures. One should diligently continue the inqui into the nature of blood constituents that a most affected by radiation, and further stud the effects of partial body irradiation. There already wide knowledge of the impairment the blood clotting mechanism (41). In additio Rosenthal (42) recently reported opalescence i the blood serum of rabbits which showed hig correlation with subsequent death of the anima due to radiation effects. The Donner Laborator group (43) has identified the molecules causin opalescence as a group of abnormal lipoproteins Very recently Jacobson *et al.* (38) have demon strated that lead-shielded spleens partially pro tect rats from lethal effects of radiation even i the circulation of the shielded spleen is clampe off during exposure to x-rays, and released soo after exposure. Further they showed that trans plantation of normal baby rat spleen into th peritoneal cavity of whole body irradiated rat also gave significant protection. This 'therapeuti influence seems to originate from humoral agent secreted from the spleen.

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AMERICAN PHYSIOLOGICAL SOCIETY

*Symposium on Demand and Supply of Energy for Function**

Chairman: JOHN O. HUTCHENS

THERMODYNAMIC PRINCIPLES AS APPLIED TO THE ANALYSIS OF BIOLOGICAL SYSTEMS[1]

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AMONG the general purposes of this symposium is the discussion of the free energy (or work) requirements of certain biological processes and the examination of the relation between such requirements and the free energy made available by spontaneous, metabolic processes.

It is the particular purpose of this paper, in addition to furnishing a brief thermodynamic background, to formulate the above stated problem, to make clear certain concepts encountered in such formulations, to discuss the form which the laws of thermodynamics must take when applied to the kind of system met with in biology and to acknowledge (with no pretense of here solving in detail) problems which arise from the inherent nature of biological systems and in the statement of the general problem posed in this symposium.

It is clear that the above outlined objectives and the constraints of reasonable space are incompatible unless some other condition is imposed. It might then be anticipated that treatment here will be brief and include frequent reference to published and forthcoming work. Any attempt to avoid formal development is doomed from the start and would result in an unbearable clumsiness of expression. The attempt has been made, however, to include only such formalism as is demanded by reasonable standards of rigor and by the fact that physical intuition is no safe guide in all situations.

1. SECOND LAW AND CRITERIA OF SPONTANEITY

It will be recalled that the First Law of Thermodynamics is a statement of the conservation of energy in which recognition is taken of the equivalence of heat and work. The usual statement is

$$dE = dQ - dW \qquad (1)$$

where dE is the increase in internal energy of the system, dQ the increment of heat absorbed *by* the system from the environment and dW is the increment of work done *by* the system *on* the environment. Sight should not be lost of the fact that dW is external work and is zero unless provisions are made for the system to perform work against external forces. The First Law places no limitations upon the transformation of energy from one form to another but demands only that energy be conserved.

On the other hand, the Second Law imposes a limitation upon the interconvertability of energy. The statements of this limitation are many and familiar (1–3). They are all equivalent, as can be demonstrated (2, pp. 127–9; 3, pp. 31–35), to this statement: No process can occur the *only* result of which is the conversion into an equivalent amount of work of heat extracted from a source which is at uniform temperature throughout. This statement, based upon invariant human experience, completes the inductive stage of thermodynamics. The law is then stated in the form of an axiom and the methods of logic and mathematics are brought into play to develop the consequences, producing an assemblage of theorems or propositions. These comprise the well studied array of formulae, equations, etc., of thermodynamics which cover a vast range of physical situations and are of remarkable generality. This department, in some ways simplest of all, is the so-called pure branch of thermodynamics.

The usual point of departure in discussing the consequences of the Second Law is the statement

$$T\,dS \geqq dQ \qquad (2)$$

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where dS is the change in entropy of the system and T is the absolute temperature at which the increment of heat dQ is absorbed. The equality sign in *equation 2* holds for a reversible process, the inequality for a spontaneous or irreversible process. If the system is isolated such that $dQ = 0$, *equation 2* becomes

$$dS \geqq 0 \qquad (3)$$

which states that the entropy of an *isolated* system can never decrease. If the process is reversible, the equality in *equation 3* holds and the system suffers no change in entropy. It must be remembered that *equation 3* applies to an isolated or adiabatic system for it is possible, with the aid of an external system, to decrease the entropy of a given system. The conditions under which *equation 3* applies may be achieved by isolating the system in a manner such that its energy is fixed and it can do no work upon the environment. For example, if the system can perform only pressure-volume work (PV work) then *equations 1* and *2* show

$$dS_{E,V} \geqq 0 \qquad (4)$$

thus the entropy at constant energy and volume can never decrease. The system can in principle always be so isolated by 'including the environment.' Thus the entropy of the system plus environment never decreases; as frequently stated —the entropy of the universe increases as a result of an irreversible process. By 'universe' is meant the system and that part of the outside world with which it may interact. A process which occurs under such conditions of isolation is plainly spontaneous in that it occurs of itself, i.e. with no external aid.

Now the conditions under which the entropy change may be used as a criterion of spontaneity are difficult to achieve and almost never dealt with in laboratory practice. The usual experimental conditions are constant temperature and pressure or, occasionally, constant enthalpy and pressure (e.g. in constant pressure, adiabatic calorimetry, $dQ = 0$). The most useful function will then be the (Gibbs) free energy, F, defined by

$$F = H - TS \qquad (5)$$

where

$$H = E + PV \qquad (6)$$

is the enthalpy or "heat content" and P and V the pressure and volume. If we write the work as the sum of PV work and work of other kinds, then for a reversible process

$$dW_r = dW'_r + P\,dV \qquad (7)$$

where dW'_r will include reversible electrical, length-tension, etc., work. It follows from *equations 5, 6, 1* and *2* that for a reversible process at constant temperature and pressure,

$$dF_{T,P} = -dW'_r \qquad (8)$$

It is a direct consequence of *equation 2* and the fact that dE is the same for a reversible and irreversible change that

$$dW_r \geqq dW \qquad (9)$$

thus

$$dF_{T,P} \leqq -dW' \qquad (10)$$

Equation 10 applies to any process at constant T and P. If $dW' = 0$, e.g. if the system can perform only PV work, then $dF \leqq 0$. If the system is allowed to do work on the environment (i.e. the environment does *not* perform work *on* the system) then dW' is positive or zero, and in this case too, $dF \leqq 0$. Thus the free energy of a system, at constant T and P, which is permitted to do work on its environment can never increase. When the free energy reaches a minimum no further change (without work *by* an external agency), can occur for this would entail an increase in free energy: the system is then in stable equilibrium. Further, according to *equation 10*, as long as the free energy can decrease, useful (non PV) work can be performed and when equilibrium is reached no further work can be performed. All of this is well known. The point which is here stressed is that the derivation of *equation 10* and the conclusions drawn from it are based squarely upon *equation 2*. We cannot insist that the entropy increase except in an isolated system. But for every statement about the entropy of an isolated system there is a corresponding statement about the free energy of the same system at constant T and P. In fact it can be shown (4), cf. SECTION 3, that the simplest manner in which to compute $dS_{E,V}$, *equation 4*, is by computing $dF_{T,P}$. Moreover it can similarly be shown (5; cf. 6 pp. 62–3) that the straightforward and simplest calculation of the entropy change at constant H and P (under which condition T changes as a result of the process) hinges upon the calculation or knowledge of $dF_{T,P}$. The 'simplicity' in these cases is due to the agreeable properties of the chemical potential.

In view of these last remarks and the role of the free energy change in the assessment of dissipation of energy in irreversible processes (SECTION 3) we now turn to a discussion of

2. FREE ENERGY CHANGE OF CHEMICAL REACTION AND TRANSPORT

For the usual[2] chemical system the free energy is completely specified when T, P and the number of moles of each chemical species are prescribed. This evidently requires that T and P be uniform throughout the system. Even when this condition is satisfied additional information is needed if the system is spatially distributed (4, pp. 96–8). The statements in what follows immediately can be modified for spatially distributed, open systems with no essential change in principle. Denote by n_k the number of moles and by μ_k the partial molal free energy (or chemical potential) of the kth substance. The free energy is given by

$$F = n_1\mu_1 + n_2\mu_2 + \cdots = \sum_k \mu_k n_k \qquad (11)$$

The chemical potentials μ_k, will be taken as

$$\mu_k = \mu_k^0 + RT\ ln\ C_k \qquad (12)$$

where R is the molar gas constant, C_k the molar concentration and μ_k^0 the so-called standard chemical potential of the kth substance. The μ_k^0 are functions of T, P and the nature of the solvent. *Equation 12* embodies all of the assumptions[3] regarding perfect, *dilute* solutes. Consider the chemical reaction

$$m_1A_1 + m_2A_2 + \cdots \leftrightharpoons m_iA_i + m_jA_j + \cdots$$

in which, stoichiometrically, m_1 moles of A_1, m_2 moles of A_2 etc. react to give m_i moles of A_i, etc. If we adopt the convention that the m's are assigned *negative* values for *reactants* and positive values for products then

$$dn_k = m_k\,dN \qquad (13)$$

where N is the "degree of advancement of the reaction" (9) and dn_k the number of moles of substance k *produced* when the reaction proceeds by the amount dN. The composition of the system is describable in terms of the initial number of moles, n_k^0, and the single parameter, N. With no loss in generality we assume that initially $N = 0$, then

$$n_k = n_k^0 + m_kN \qquad (14)$$

If F_2 and F_1 are the values of free energy in the final and initial states in which the concentrations are C_k and C_k^0 respectively, then it follows straightforwardly from *equations 11*, *12* and *14* that $F_2 - F_1 = \Delta F$ is given by

$$\Delta F = N\{\Delta F^0 + RT \sum_k m_k\ ln\ C_k^0\} + \sum_k (n_k^0 + m_kN)\ RT\ ln\ \frac{C_k}{C_k^0} \qquad (15)$$

or

$$\Delta F = N\{\Delta F^0 + RT \sum_k m_k\ ln\ C_k\} + \sum_k n_k^0\ RT\ ln\ \frac{C_k}{C_k^0} \qquad (16)$$

Where ΔF^0 is the standard free energy change. In *equation 15* the quantity in brackets gives the free energy change, per equivalent,[4] at the *initial* concentration; the remaining terms give the free energy change of adjusting the final number of moles, $n_k^0 + m_kN$, from initial to final concentrations. Similar remarks apply to *equation 16*. In spite of complications which may arise in applying *equation 15*, or a modification of it, to biological systems a fortunate, simplifying condition is frequently met in practice (10). Evident from *equation 15* is the fact that if some reactants are completely consumed so that the final number of moles is zero and if the concentrations of some reactants do not change and if, further, the concentrations of none of the products change, then the terms in *equation 15* associated with the changes in concentration vanish (e.g., substances such as O_2 and CO_2 are under usual conditions present at constant concentration; products such as starch, glycogen and fat are present at constant, saturated, concentration, and added substrates such as acetate, ethanol, etc. are completely consumed). It can be shown (11) that rather restrictive numerical limitations can be placed upon the terms, in *equations 15* and *16*, associated with concentration changes and numerous special cases can be discussed.

If many reactions are proceeding, as is usually the case in practice, then the extension of the above discussion is as follows: Let N_j be the degree of advancement of the jth reaction and

[2] We must exclude strained elastic solids, systems in which surface energy is an appreciable part of the total, etc.

[3] In particular *equation 12* implies (4, p. 93; 7; 8, p. 189) that there is no heat or volume of dilution.

[4] Clearly, from *equation 14*, $N = (n_k - n_k^0)/m_k$ is the number of equivalents of any reactant converted or of any product formed.

m_{kj} the stoichiometric coefficient of substance k in the jth reaction. Then

$$\left.\begin{aligned} dn_k &= \sum_j m_{kj}\, dN_j \\ n_k &= n_k^0 + \sum_j m_{kj}\, N_j \end{aligned}\right\} \qquad (17)$$

For the total change in free energy one then obtains a sum of expressions, each of which is similar to *equation 15* or *16*. This causes no difficulty. However, when, and this is the rule in biological systems, certain substances participate simultaneously in several reactions some special discussion (see SECTION 4) is needed for the calculation of 'efficiency.'

Finally it follows (12) from *equations 11, 12, 13* that the time rate of change of the free energy is given by

$$\frac{dF}{dt} = \sum_k \mu_k m_k \frac{dN}{dt} \qquad (18)$$

where dN/dt is simply related to the usual chemical kinetic expression for the reaction rate. If consecutive reactions, branched or coupled, are involved the summation is extended over all steps involved (12).

Consider a system consisting of two uniform phases. Let n_1^0 and n_2^0 be the initial number of moles of a given solute and C_1^0 and C_2^0 the initial concentrations in the phases. If n moles are transferred from *1* to *2*, the free energy change is

$$\Delta F = nRT\, ln\, \frac{C_2^0}{C_1^0} + (n_1^0 - n)\, RT\, ln\, \frac{C_1}{C_1^0} + (n_2^0 + n)\, RT\, ln\, \frac{C_2}{C_2^0} \qquad (19)$$

where C_1 and C_2 are the final concentrations and it has been assumed that the standard chemical potential is the same in the two phases. If several solutes are transferred the total ΔF is obtained by summing expressions such as *equation 19*. Strictly speaking, when ions are transferred, in addition to substituting activities for concentrations in *equation 19* there should be added a term $nz\mathfrak{F}\Delta\mathcal{E}$ where z is the valence, $\mathfrak{F}$ the Faraday and $\Delta\mathcal{E}$ the electrostatic potential difference between the phases. In summing over all species involved, however, such electrical terms must cancel (8, 13).

If the system under investigation is not spatially uniform then the treatment in this section requires modification. In the first place the free energy is no longer given by *equation 11* for the values of the μ_k depend upon the point in the system at which they are evaluated. The total free energy is now given (4) by

$$F = \int_v \sum_k \mu_k C_k\, dV \qquad (20)$$

when the μ_k and C_k are functions of the spatial coordinates and the integral is extended over the volume of the entire system. If there are gradients in concentration there will in general be flow of matter, although this is not necessarily (14) true of every solute. In any event the μ_k and C_k are determined at every point by the rates of diffusion, of chemical reaction and of flow of matter across the surface of the system. The value of F may not be changing with time, as is the case (4) in the steady state, but the value of *equation 20* is still determined by the *rates* at which processes occur within the system. Here we see an important example of the interplay of 'thermodynamics proper' and *rates*. The time rate of change of F for a spatially distributed, open system is simple to obtain formally, often difficult to apply. It can be shown (4) that

$$\frac{dF}{dt} = \int_v \left\{ J_x \frac{\partial \mu}{\partial x} + J_y \frac{\partial \mu}{\partial y} + J_z \frac{\partial \mu}{\partial z} \right\} dV + \int_v \mu q\, dV + \int_s J_s \mu\, ds \qquad (21)$$

where J_x is the flow, per unit area, per unit time, in the x-direction and similarly for J_y and J_z; q is the rate of production, per unit volume; J_s is the flow in the direction perpendicular to the surface, s, of the system. *Equation 21* has been written for *one solute:* such expressions for all solutes are summed in the general case. The first integral gives the contribution from the flow or transport of substances in the system due to the fact that gradients exist; the second integral is the contribution due to chemical reaction and when q is expressed according to chemical kinetics the integrand is a sum similar to *equation 18*; the last integral is the contribution due to the flow of matter, across the surface, into the system. An important special case is the evaluation of the first integral in *equation 21* for the case of a region (cell) in which concentrations are uniform, separated by a barrier (membrane) from another region (environment or extracellular space) which is likewise uniform. There will be no gradients except the 'concentration drop' across the membrane. We may assign the membrane a finite thickness, attribute to it the properties of a

bulk phase[5]; or take it in the limit as infinitesimal; or plead complete ignorance of the dimensions and properties of the barrier. The appropriate expression can be shown (4) to be

$$\left(\frac{dF}{dt}\right)_{\text{diff.}} = A \cdot J \cdot RT \ln \frac{C_0}{C_i} \qquad (22)$$

where A is the area of the membrane, J the flow (per unit area and time) out of the cell and C_0 and C_i are the concentrations outside and inside the cell respectively. The product AJ is the total flow (moles/time) out of the cell and (13) may be evaluated empirically without reference to the formal law of flow across the membrane. *Equation 22* is for a single solute and embodies the assumptions discussed for *equation 19*. It should be mentioned that if concentrations are not uniform within the cell then additional terms, frequently of the same order of magnitude as *equation 22* are contributed. The use of *equation 22* may then be a fairly rough approximation but the error is not easily evaluated.[6]

3. FREE ENERGY, DISSIPATION OF ENERGY AND AVAILABLE WORK

According to the Second Law, the entropy, (unlike energy, volume, etc.), is not conserved when irreversible processes take place. From what has been said in SECTION 1, the entropy gain of a system is not equal and opposite to the entropy loss of the environment unless the process is reversible. If the process is irreversible, we are assured by the Second Law that the entropy of the system plus environment increases. Irreversible processes thus result in the creation of entropy. This is in essence the basis of the equality statement of the Second Law (9, 6, 17, 4). It may thus be stated that

$$TdS = dQ + dQ^* \qquad (23)$$

where dQ^* is the so-called uncompensated heat (9, 17). *Equation 23* in conjunction with the inequality *equation 2* shows that

$$dQ^* \geqq 0 \qquad (24)$$

Further,

$$dS = dS_r + dS^* \qquad (25)$$

where dS_r is the entropy reversibly communicated to the system from the environment and dS^* is the entropy *produced* by irreversible processes which occur in the system, and is given by

$$T\,dS^* = dQ^* \geqq 0 \qquad (26)$$

The *total* entropy of a system does not necessarily increase but the irreversible production of entropy is positive or zero.

If one considers a system at constant energy and volume the entropy change for such a system must correspond to the irreversible production of entropy. For a process which can perform only PV work it can be shown (4) that

$$dS_{E,V} = -\frac{dF_{T,P}}{T} = dS^* \geqq 0 \qquad (27)$$

or for a finite change

$$\Delta S^* = -\frac{\Delta F_{T,P}}{T} \qquad (28)$$

The physical significance of the irreversible production may be stated as follows. It can be shown that $T\,dS^*$, or $T\Delta S^*$ for a finite change, represents the dissipation or 'degradation' of energy which accompanies an irreversible process, i.e., this quantity represents the amount of energy rendered unavailable for useful work as a result of irreversibility. It is clear then that the irreversible production of entropy represents, in the above sense, the waste or inefficiency of the process in question; a matter with which accompanying papers (10, 13) are concerned. From *equations 1*, *23* and *26* it follows that

$$dE - T\,dS = -T\,dS^* - dW \qquad (29)$$

Since, cf. discussion in (17) dE and dS depend only upon the initial and final states, the quantity $T\,dS^* + dW$ has a set value: If $T\,dS^*$ decreases dW increases correspondingly. *Equations 1*, *23* and *26* likewise imply that

$$dW = dW_r - T\,dS^* \qquad (30)$$

which shows $T\,dS^*$ to be an amount of work which could have been performed had the process been reversible or the difference between the maximum possible work, dW_r, and the actual work, dW, in the irreversible process.

It can be shown (5) that for a process which can perform non PV work, that

$$dS_{E,V} = dS^* = -\frac{dF_{T,P} + dW'}{T} \geqq 0 \qquad (31)$$

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[5] This will lead to the usual permeability expressions (14–16).

[6] To make the comparison between contributions from gradients inside and from the concentration difference across the membrane one must assign a permeability coefficient, a diffusion coefficient and dimensions of the cell. The results clearly depend upon these choices.

from which

$$dW' = -dF_{T,P} - T\,dS^* \qquad (32)$$

Equation 32 furnishes the *equality* statement corresponding to *equation 10*. It also states in view of *equation 8*, that the useful (non PV) work is the maximum or reversible work minus the energy dissipated.

It is difficult to exaggerate the simplicity of *equations 27* and *31* and their derivations (4, 5) when comparison is made to the general formulation of the irreversible production of entropy (18) or the detailed derivation for special cases (6, 19) to which these results apply. We are in a position to write down at once the rate of dissipation of energy

$$T\frac{dS^*}{dt} = -\frac{dF}{dt} \qquad (33)$$

for diffusion[7] or chemical reaction by substituting *equation 22* or *18* respectively into *equation 33* and correspondingly for the cases of finite changes. It is easily shown (4) directly from the simple laws of diffusion that the rate of dissipation due to diffusion is the rate of *work* done against the forces of resistance to flow.

It is sometimes stated and often implied (e.g. 20–22) that the free energy dissipated in irreversible processes or the free energy not 'harnessed' or the energy wasted appears as heat. It is clear from the foregoing that this is not correct. The *actual* heat is related to the energy dissipated by *equation 23*. For a spontaneous chemical reaction the dissipation is numerically equal to the free energy change. In the special case of constant temperature and pressure when $\Delta S = 0$ then the dissipation *is* given by the heat evolved, viz., $-\Delta H$; which further implies that $\Delta F = \Delta H$ which is known not to be true in general. In the case of diffusion the dissipation is again numerically equal to the free energy change. If the solute is perfect[2] there is no heat change.

It is not legitimate to regard heat evolved as 'waste energy' nor does dissipated energy *necessarily* appear as heat.

4. CALCULATION OF EFFICIENCY

Consider an overall process which is spontaneous and which proceeds with a free energy change ΔF. Let it be capable formally of *representation* as the 'sum' of two component processes with associated free energy changes ΔF and ΔF_2. Then, since free energies are additive

$$\Delta F_1 + \Delta F_2 = \Delta F. \qquad (34)$$

Assume now for definiteness that ΔF_1 is positive and ΔF_2 negative. It will then be said that the second process 'drives' the first, and further, that the free energy 'available' from the second process is $-\Delta F_2$ and that 'used' in the first is ΔF_1. The ratio

$$X = \frac{\Delta F_1}{-\Delta F_2} \qquad (35)$$

is called the 'efficiency', i.e., the efficiency in the overall or 'coupled' process of the utilization of free energy made available by a spontaneous process in driving a non-spontaneous process. This quantity, which we will refer to hereafter as simply the efficiency, is also called the 'machine efficiency' (20, 10) and, more properly, the 'thermodynamic efficiency' (23).

The calculation and interpretation of efficiency as defined above requires at most what may be an inordinate amount of knowledge about the process and system and at least great care and thorough qualification in stating what is meant. Only in the simplest cases is it possible to *unambiguously* identify the quantities in *equation 35* and assign them the meanings discussed above. In the remainder of this section the various factors and difficulties involved in the calculation of efficiency will be discussed. First, however, some remarks on the definition given in *equation 35* will be made. The 'machine efficiency' was first discussed (20) in connection with the problem of whether or not organisms operate within the Second Law. It is easily shown from *equations 34* and *35* that an efficiency of more than 100 per cent ($X > 1$) implies that ΔF is positive. It is well known that the effect of 'coupling' of processes is one of conservation of free energy. This is implicit in the definition of X, since[8]

$$X = 1 - \frac{\Delta F}{\Delta F_2} \qquad (36)$$

Thus the smaller the loss in free energy, ΔF, the greater the efficiency. The term 'machine efficiency' is somewhat misleading since in many cases no external work is involved (e.g., expenditure of free energy in maintaining concentration gradients, chemical synthesis, etc.). The

[7] In *general* the dissipation due to diffusion is given by the first integral in *equation 21* preceded by a minus sign.

[8] This equation shows that, since ΔF_2 is negative, if ΔF is positive X exceeds unity.

use of this term for X as defined above may be clarified as follows. If the non-spontaneous component process is to occur alone this is possible (*equation 8*) only if some external agency does work in amount at least ΔF_1 upon the system. When the spontaneous component process occurs it is *possible* for the system to perform work in amount $-\Delta F_2$ upon some external agency. The efficiency is the ratio of these theroretical works, although in the actual coupled process it may be that no external work is performed. Finally, it can be shown that, in terms of the concepts discussed in SECTION 3, X is a measure of energy dissipated. From *equations 27* and *36* it follows that

$$X = 1 - \frac{T\Delta S^*}{-\Delta F_2} \qquad (37)$$

which states that the efficiency is unity minus the fraction of the (available) energy which is dissipated.

a) Component processes: For the simple process

$$ATP + \text{Glucose} \leftrightharpoons \text{Glucose - } P + ADP$$

assume that conditions are such that

$$P + \text{Glucose} \leftrightharpoons \text{Glucose - } P \qquad \Delta F_1 > 0$$
$$ATP \leftrightharpoons ADP + P \qquad \Delta F_2 < 0$$

There is no question here that the component processes are those shown. In more complicated cases, however, (10) the overall process may be partitioned into component processes in several ways. Each partition leads to a different ratio of free energies and hence a different efficiency. The choice of component processes is usually clear from known pathways of intermediary metabolism. These problems are discussed elsewhere (10).

b) Assignment of ΔF to a given reaction: A problem closely related to the one above is the following. The substance A is converted simultaneously to substances B and C.

$$A \leftrightharpoons B$$
$$A \leftrightharpoons C$$

The calculation of the *total* free energy change is simple by the method already discussed (SECTION 2). We wish however to calculate the free energy change due *only* to the conversion of A to B. The concentration of A at any stage of the reaction is determined by how much A has been converted over *both* paths. The increment of free energy due to converting a small amount of A to B will then depend upon the amount of A which has been converted over both paths. Clearly the quantity which we wish to compute depends upon the relative *rates* of the two reactions. If we define $f_1(t)$ and $f_2(t)$ as the number of moles of A which have at time t been converted to B and C respectively then it can be shown (11) that

$$\Delta F(A \rightarrow B) = \int_0^t \left\{ \Delta F^0 + RT \ln \frac{[B]}{[A]} \right\} \frac{\partial f_1}{\partial t} dt \qquad (38)$$

is the desired quantity where $[B]$ and $[A]$ are the concentrations of B and A at any time. If n_A^0 and n_B^0 are the initial number of moles of A and B, *equation 38* gives

$$\Delta F(A \rightarrow B) = f_1 \left(\Delta F^0 + RT \ln \frac{[B]}{[A]} \right) + n_B^0 RT \ln \frac{[B]}{[B]_0} + n_A^0 RT \ln \frac{[A]}{[A]_0} \qquad (39)$$
$$+ RT \int_0^t \frac{n_A^0 \, df_2 + f_2 \, df_1 - f_1 \, df_2}{n_A^0 - f_1 - f_2}$$

The terms in *equation 39*, except the integral, are identical with the terms obtained when *equation 16* is applied to the reaction in question. It can be shown that if the functions f_1 and f_2 are proportional, the last two terms in *equation 39* give

$$\frac{f_1}{f_1 + f_2} n_A^0 RT \ln \frac{[A]}{[A]_0}$$

Thus the usual term in *equction 16* contributed by the change in concentration in A is multiplied by the fractional amount of A converted to B. The problem can in principle be solved whenever the rates are specified whether they are empirically determined or otherwise known. In general such simple results as the above cannot be expected. This problem, illustrated by the very simple example above, is a prominent feature of the calculation of 'efficiency of synthesis' in which, for example, a substrate is simultaneously oxidized and assimilated.

c) Available free energy: Perhaps the most vexing problem to be encountered in the calculation of efficiencies is that of deciding the amount of free energy 'available' for driving a given process. At least account must be taken of the resting, non-nutrient or maintenance level of metabolism. If the available free energy is taken as that available from spontaneous processes above and beyond the maintenance level (10) it is then assumed that the cost of maintenance is not altered by the activity or synthesis in

question. Certainly circumstances can often be achieved wherein the 'correction' for resting or non-nutrient metabolism is negligible (10). But, in principle, one must entertain the idea that the cost of maintenance is not invariant under imposed conditions of activity. While, in the light of present knowledge, it cannot be asserted that activity and resting metabolism are even qualitatively the same it is probable that they are not utterly independent. As discussed elsewhere (12) it can be shown that various processes subserved by common energy-rich intermediates compete for such compounds. Thus the enhancement of a given process can suppress others with which it competes and conversely. If maintenance processes are not 100 per cent efficient, and this must be granted, then it is possible to divert energy from maintenance pathways without actually impairing maintenance.

The real problem here is that of identifying what may be called 'unavoidable waste.' We know that for spontaneous processes, processes proceeding at a finite rate, there must be dissipation. But what now is unavoidable waste?

Consider for example that among the processes competing for energy-rich intermediates there occurs wasteful splitting. It is known that such occurs and is even catalyzed by specific enzymes. Now the cell makes best use of available energy when the useful processes compete most successfully with wasteful splitting. This is governed by relative rates and those processes which are among the most successful competers are most efficient. If wasteful splitting occurs this is just the type of information we wish to have reflected in an efficiency calculation. This is bad management on the part of the cell.

On the other hand consider the following situation. In computing the free energy of spontaneous processes if the calculation is made for the concentrations existing in the medium it is then assumed that a given amount of free energy is available. It is known that in general, but not necessarily (14), the concentration of a consumed substance is *lower* in the cell than in the medium. Calculation at the cellular concentration yields a different free energy. Moreover (*equations 21* and *22*) the difference corresponds to dissipation in the diffusion field. In general we would insist that this free energy is wasted insofar as syntheses are concerned. This is poor architecture on the part of the cell; open, non-uniform systems are naturally inefficient; this is unavoidable waste consequent upon the structured nature of the system. But all of this is true if we are interested in a given synthesis and nothing else. It has been shown (4, 14) that as a result of the metabolism of substrates, non-metabolized solutes can be maintained at a higher concentration inside the cell than in the medium. More significantly, the cost of this maintenance is derived from free energy of the diffusion field of metabolized solutes. We cannot then regard the free energy of diffusion as wasted to the *total* economy of the cell. Conversely if the total free energy derived from metabolized solutes be regarded as available for maintaining concentration differences, the computed efficiency of this maintenance is quite low and under the circumstances discussed (4, 14) it is known that it is the free energy of diffusion alone which is responsible for this maintenance. The rates of free energy change due to diffusion and reaction are (*equation 21*) inseparably linked and assignment of free energy available for separate processes is often difficult.

It has also been shown (14) that soluble catalysts will distribute in a definite manner in the diffusion field of the cell. Often this distribution is such that the rates of catalyzed reactions are different (either greater *or* less) than would be the case if the same number of moles of reactant and catalyst were placed in a closed uniform system of equal volume. Such distributions are maintained by the diffusion free energy and, in addition to being regulatory devices, contribute to the cost of maintenance as certainly as do more obvious maintenances of structural integrity. An important feature is that such distributions and hence the cost of maintenance are shifted when new conditions are imposed upon the cell. It is a direct consequence of the general laws of diffusion and kinetics (14) that the rate at which a given process occurs is dependent upon the rates of other, chemically *independent*, reactions. The efficiency of a given synthesis will then be different when other syntheses are occurring.

Further examples cannot be discussed here. It should be clear from the foregoing that although efficiency calculations are based upon classical thermodynamics, often certain extra-thermodynamic information is required. In particular, rates, nature and concentration of intermediates and 'geometry' or structure play a major role in determining how well the cell can dispose of available energy.

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FACTORS IN NERVE FUNCTIONING

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RECENT developments emphasize a close relation between nerve activity and ion movement. Hodgkin and his associates have presented the evidence in favor of their view that spike production is related to sodium entry; this is believed to be followed closely by the known exit of potassium (1–4). Keynes (5, 6) has obtained direct analytical data for sodium influx as well as for the previously established potassium escape. As recognized by these investigators, the data are either highly indirect and dependent on a number of assumptions or seriously dissociated in time from the bioelectrical events assumed to be involved.

Earlier studies (7–12) have led to the conclusion that slow changes in the polarization of both frog and crab nerve, under a large variety of conditions, are caused primarily by alterations in the potassium concentration at the fiber surface which result from the release and active uptake of potassium. If this were substantiated one would have at hand the opportunity to study and to correlate electrical phenomena simultaneously with ionic transfer and thereby to evaluate concisely the factors governing the kinetics and energetics of the system.

The purposes of this report are *a*) to describe briefly recent results of the study of potassium and sodium transfer in relation to the earlier bioelectrical observations, *b*) to provide a more precise approach to the kinetics of ionic and electrical phenomena for comparison with available data, and *c*) to employ these principles for a preliminary survey of the energetics of the resting and active state. Full details will be given elsewhere.

RESULTS

Anoxia. BIOELECTRICAL OBSERVATIONS. Oxygen lack causes progressive depolarization which is slowed by glucose but accentuated by iodoacetate poisoning whether glucose is present or not (7, 11–15). Return to oxygen leads to repolarization, usually with overshooting (7, 11, 15, 16); in glucose the amplitude of repolarization is less (11,12).

ANALYTICAL DATA. Almost simultaneously evidence was reported for frog and crab nerve (17, 18) that anoxia causes potassium release, thereby refuting earlier claims to the contrary (19, 20); reabsorption was noted during the period of oxidative recovery. Glucose was found to reduce the ionic shifts in invertebrate nerve (18).

More details have now been obtained for frog nerve. Table 1 confirms the finding by Fenn and Gerschman that anoxia causes potassium release. In addition it demonstrates that glucose retards this loss and that iodoacetate poisoning inhibits the sugar effect. Because of its importance for the theoretical development later, mention must be made of our failure to confirm the conclusion by Fenn and Gerschman (17) that potassium release is not a continuous process throughout anoxia. Our experiments, now numbering in the hundreds, involve repeated analyses of the media from the same nerves as well as of the nerves themselves; none has failed to demonstrate a continuing loss of potassium.

Table 2 illustrates that removal of the external sheath improves potassium escape during anoxia only slightly but is essential for a demonstration of potassium uptake during a single hour of recovery. The unimportance of the sheath for the anoxic release indicates that another boundary, such as the individual fiber membranes, may be limiting potassium movement under these conditions. When potassium shifts are rapid, as may be the case during recovery or during stimulation, the epineurium would be expected to be the limiting factor.

Table 3 summarizes sodium and potassium data obtained by analyses of the nerves immediately following anoxia. In general, the escape of potassium is accompanied by an uptake of sodium, the latter being greater as described for stimulation (6). The protective action of glucose extends to sodium as well as to potassium. Within the error of our measurements of dry and wet weights, water movement usually was not significant.

Veratrine and Cocaine. BIOELECTRICAL OBSERVATIONS. These alkaloids have opposite effects

on the rate of anoxic depolarization; the anesthetic decreases, the 'unstabilizer' increases it (9, 11, 12, 21). Many other compounds (22), including procaine and antihistaminics (12, 21), duplicate the action of cocaine and may be called 'stabilizers'; at physiological concentrations, such as employed here, no respiratory effects are demonstrable (23). Veratrine, however, increases the respiratory rate (24). The antagonism between veratrine and cocaine is shown particularly clearly by the prevention of the depolarizing action of higher concentrations of the alkaloid mixture by low concentrations of the stabilizer (12).

TABLE 1. POTASSIUM LOST IN N_2 TO RINGER[1] CONTAINING THE INDICATED CONSTITUENTS

R. pipiens

HOURS IN N_2	SUC.	GLUC.	IAA + GLUC.	DIFF.[2]
5	10.8 ± 0.5[3]	7.0 ± 0.5[3]		3.8 ± 0.2
2		0.7 ± 0.4[4]	2.7 ± 0.3[4]	1.97 ± 0.3

Data in μM/gm. wet weight.

Suc = sucrose, Gluc = glucose, IAA = iodoacetate. Variability in this and all following tables expressed as the standard error based on at least 6 paired nerves.

[1] Composition as previously noted (7); potassium concentration 1.7 μM/ml. [2] Standard errors computed from differences of paired nerves. [3] Sugar concentration 10 μM/ml. [4] Glucose concentration 20 μM/ml., IAA concentration 1 μM/ml.

TABLE 2. POTASSIUM LOST DURING ANOXIA AND THE FOLLOWING HOUR IN OXYGEN BY INTACT (I) AND DESHEATHED (D) NERVES

R. pipiens and R. catesbiana

	2 HR. N_2 LOSS		1 HR. O_2 LOSS	
	I	D	I	D
	1.3	2.2	0.24	−0.85[1]
D–I...	0.9 ± 0.3		−1.1 ± 0.11[1]	

Data in μM/gm. wet weight.

[1] Potassium absorbed from medium.

ANALYTICAL DATA. Figure 1*A* illustrates *a*) the increased escape of potassium in veratrine concentrations which cause depolarization and *b*) its complete prevention by cocaine in concentrations which prevent veratrine depolarization. Figure 1*B* demonstrates the ability of cocaine to block veratrine action even after the alkaloid mixture has acted for some time; the cessation of potassium release in this case is followed by a significant absorption. Table 4 provides typical data showing the increased release of potassium during anoxia in veratrine concentrations too low to cause either depolarization or potassium release in oxygen. Table 5 shows the behavior of both sodium and potassium. As in anoxia, sodium entry usually exceeds potassium exit in veratrine; both are prevented by cocaine. The anesthetic also reduces sodium and potassium transfer during anoxia, but its effectiveness is substantially less than in veratrine. In all of these cases water shifts were not significantly greater than the experimental error of the weight determinations used for their measurement.

Stimulation. BIOELECTRICAL OBSERVATIONS. The after-potentials of crab nerve are particularly suited for correlation with other less accessible characteristics because of their typical slowness. Under special conditions the well-known depolarization during a tetanus (14) is followed by recovery leading to an overshooting of polarization level which apparently represents a positive after-potential (11). The relation of the depolarization to the negative after-potential is shown by its increase with veratrine; the amplitude of the repolarization is correspondingly greater (11, 25).

ANALYTICAL DATA. These were obtained as in most of the previous experiments by analyses of small volumes of media recirculated past the nerves and collected at regular intervals; the perfusion units employed permitted concomitant observation of the spikes with stimulation. It has been found that in addition to the potassium escape long known to accompany activity, potassium reabsorption occurs during recovery; moreover, these shifts, like the corresponding electrical changes, are accentuated by veratrine (25).

In frog nerve potassium release also results from stimulation (26). The earlier work has been considered questionable because rather extreme conditions of stimulation were necessary. Table 6 illustrates the considerable increase in the potassium released per impulse when the external sheath of bullfrog nerve is removed. Milder stimulation, practicable in the absence of the sheath, gives about the same loss per impulse as a more rigorous tetanus. As in crab nerve, low veratrine concentrations increase potassium es-

cape during tetani. Potassium uptake following stimulation has not been seen in this tissue; however this may have been obscured by the potassium loss which occurs from *R. catesbiana* nerve, whether sheathed or desheathed, even when at rest in oxygen.

Studies restricted to desheathed fibers must be regarded as tentative pending the evaluation of the significance of the considerable swelling they undergo: in 3 to 4 hours their weight increase approaches a maximum of 30 per cent relative to the initial weight with sheath, and of 40 per cent relative to that without sheath. Sodium is also gained in amounts equivalent to the entry of isotonic saline. These effects may be extrafibrillar for *a*) the potassium content relative to the dry weight is little changed, *b*) the spike heights and the ability to withstand repetitive activity are not affected significantly, and *c*) the improved absorption of potassium following anoxia is hardly attributable to damage. Fortunately, invertebrate and intact vertebrate nerve provide a check on the results with desheathed fibers.

In view of the data which have been presented, the conclusion appears unavoidable that potassium shifts—and corresponding exchanges by sodium where these have been followed—are intimately correlated both in direction and time with the after-potentials and the changes in resting potential.

Table 3. Sodium and potassium contents of nerves, paired with respect to indicated conditions, after 6-hour exposure time

R. pipiens

	Na		K	
	N_2	O_2	N_2	O_2
N_2-O_2	82.0 ± 2.3	66.3 ± 1.7	27.1 ± 1.3	38.4 ± 1.2
	15.7 ± 1.5		−11.3 ± 1.3	
	N_2 + 10mM Gluc.[1]	N_2 + 10mM Suc.[1]	N_2 + 10mM Gluc.[1]	N_2 + 10mM Suc.[1]
Gluc-Suc	58.7 ± 1.8	67.9 ± 2.0	28.7 ± 1.1	25.5 ± 0.8
	−9.2 ± 3.3		3.2 ± 0.7	

Standard errors of the differences are computed from the differences of paired nerves.
Data in μM/gm. wet weight.
[1] Sugar replacing an osmotic equivalent of NaCl in the medium.

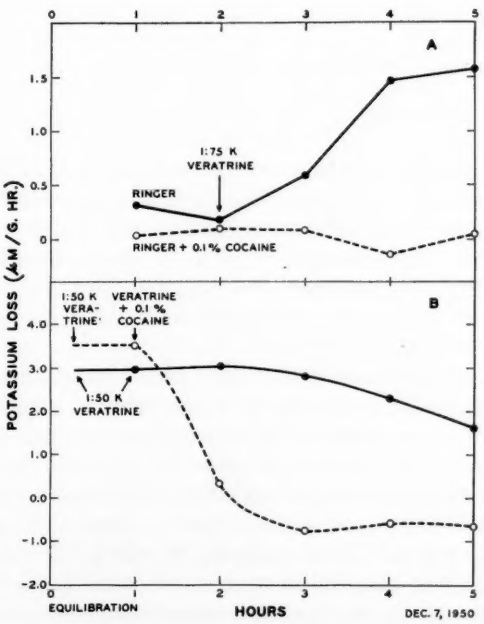

Fig. 1. *A*: Effect of veratrine on rate of potassium release by frog nerve in the presence and absence of cocaine. *B*: Rate of potassium release in veratrine compared with that following application of cocaine.

THEORY AND RELATED DATA

Sodium vs. Potassium. Bioelectrical Observations. The observed movements of potassium were predicted from the earlier electrical measurements on the assumption that accumulation of potassium in the extracellular spaces, such as would occur in the usual moist chamber situation if potassium did actually leak out, could be computed from the known depolarizing action of excess potassium when applied by way of the external medium. Although the predictability of potassium behavior is now in favor of the original hypothesis, our more recent finding that sodium

TABLE 4. COMPARISON OF POTASSIUM RELEASED HOURLY BY CONTROL (R) AND VERATRINIZED NERVES (V) SUBJECTED TO SUCCESSIVE EXPOSURES OF OXYGEN AND NITROGEN

GAS	R	V[1]	R	V[2]
O_2	0.0	0.0	0.0	0.0
O_2	0.1	0.0	0.0	0.0
N_2	1.0	4.3	1.1	3.1
N_2	1.5	4.0	1.5	3.9
O_2	−0.6	0.0	−1.0	1.7

Data in μM/gm. wet weight.
[1] 0.5 mg. % veratrine. [2] 0.17 mg. % veratrine.

potassium accumulation or from its prevention or decrease. In this connection Feng's observations on the failure of conduction during anoxia and iodoacetate treatment and the utilization of lactate during iodoacetate poisoning only when oxygen is present, parallel perfectly later findings on the state of polarization (15). This is in accord with the very early conclusion that the polarization level is an important element in conduction. The recent experiments of Lorente de Nó (16) lend further support to this view as well as to the postulate that conduction failure, where depolarization is known to occur, is an index of such

TABLE 5. SODIUM AND POTASSIUM CONTENTS OF PAIRED NERVES SUBJECTED TO DIFFERENT CONTROL (C) AND EXPERIMENTAL (X) CONDITIONS FOR 6 HOURS

R. pipiens

CONDITIONS		Na		K	
		X	C	X	C
X = N_2 + 0.1% cocaine C = N_2	X–C....	69.9 ± 1.6 −7.8 ± 2.1	77.7 ± 2.3	35.0 ± 1.8 5.2 ± 0.5	29.8 ± 1.5
X = 1:50k[1] veratrine C = Ringer	X–C....	80.9 ± 2.1 20.1 ± 2.4	60.8 ± 2.2	16.8 ± 0.8 −19.3 ± 1.2	36.1 ± 1.8
X = 1:50k[1] ver. C = ver. + 0.1% cocaine	X–C....	84.4 ± 2.2 21.9 ± 1.4	62.5 ± 1.9	20.6 ± 1.3 −16.7 ± 0.9	37.3 ± 0.5

Standard errors of the differences computed from the differences of paired nerves.
Data in μM/gm. wet weight.
[1] k = 1000.

moves in the opposite direction—particularly in the light of Ussing's suggestion (27) that sodium extrusion is the primary process responsible for frog skin potentials—raises the question as to which ion, if either, is immediately linked with metabolism and concerned with the electrical changes.

Several observations in the literature, and some which we have recently made, seem to provide definitive answers. With regard to anoxic failure, Feng and Gerard (28) reported that the mere washing of anoxic frog nerve with oxygen-free solution caused partial recovery. Feng (29) reported two observations to the effect that conduction failure occurred more quickly when nerves exposed to iodoacetate were mounted in a moist chamber than when they were kept in a large volume of the same iodoacetate Ringer. Similar observations for anoxia have recently been reported for frog nerve (30). These are obviously results such as would follow from

TABLE 6. POTASSIUM LOSS PER IMPULSE AS DETERMINED BY STIMULATION OF INTACT AND DESHEATHED BULLFROG SCIATIC NERVE, IN THE PRESENCE OR ABSENCE OF 0.05–0.1 MG. % VERATRINE

CONDITIONS OF STIMULATION	100 SHOCKS/SEC. FOR 60 MIN.	20 SHOCKS/SEC. FOR 30 MIN.	
		RINGER (R)	R + VERATRINE
Desheathed nerve	1.8×10^{-5}	2.0×10^{-5}	4.6×10^{-5}
Intact nerve	1.3×10^{-6}		

Data in μM/impulse, gm wet weight.

depolarization. His demonstration that an anodal current restores conduction and repolarizes in the continued presence of experimental agents which have been demonstrated to cause potassium release, may now be taken as evidence for the integrity of the semipermeable properties of

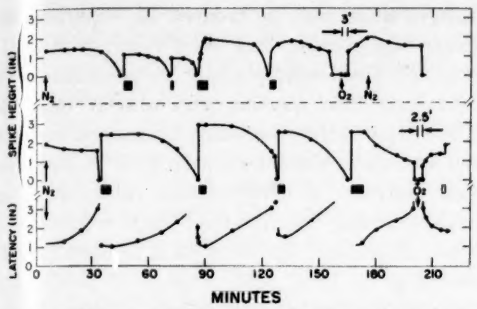

Fig. 2. TEMPORAL CHARACTERISTICS of functional changes of a squid axon, only partly cleaned, *a*) in nitrogen gas, *b*) upon washing with nitrogenated sea water ■, *c*) upon return to oxygen gas, and *d*) upon washing with oxygenated sea water □. Spike height and latency (including conduction time) in inches of oscilloscope deflection.

and conduction by washing and anodal currents appears irreconcilable with Ussing's proposal that failure of sodium extrusion leads to depolarization; conversely, the accumulation of excess extracellular potassium is in simple accord with known facts and provides a consistent explanation for the observations.

ANALYTICAL DATA. The possibility of distinguishing between a mechanism concerned with potassium uptake rather than with sodium extrusion is afforded by observations on the effects of a low sodium level in the medium on ion transfer. With the sodium concentration reduced to that of potassium, the sensitivity attained for detection of sodium shifts is almost equal to that for potassium; moreover, analyses can be carried out for both sodium and potassium on the same medium samples.

TABLE 7. POTASSIUM LOSSES TO RINGER, AND SODIUM AND POTASSIUM LOSSES TO A LOW SODIUM RINGER, MEASURED AT SUCCESSIVE INTERVALS FIRST IN OXYGEN THEN IN NITROGEN

R. pipiens

CONDITION	RINGER K LOSS	LOW SODIUM RINGER[1]	
		K LOSS	Na LOSS
1 hr. O_2	0.24 ± 0.10	0.34 ± 0.15	3.88 ± 0.19
1 hr. O_2	0.08 ± 0.11	0.31 ± 0.13	2.49 ± 0.25
2 hr. N_2	2.02 ± 0.19	2.13 ± 0.20	1.91 ± 0.18[2]
2 hr. N_2	3.50 ± 0.15	2.58 ± 0.19	1.05 ± 0.24[3]

Data in μM/gm. wet weight.
[1] NaCl replaced with sucrose or choline chloride. [2] The expected sodium loss calculated from oxygen data is 2.10 μM/gm. [3] The expected sodium loss calculated from oxygen data is 0.86 μM/gm.

the fiber membranes and for the transport back into the fibers by the current of the potassium which had leaked into the surrounding space.

The effects of washing have been examined in a system where barriers to electrolyte transfer are close to a minimum—the nerves and giant axons of the squid. Figure 2 illustrates the dramatic effects noted for the action potential of a partially cleaned giant axon. Washing with oxygen-free solution, after failure in nitrogen gas, restored the spike height and normal conduction velocity practically instantaneously and this could be repeated many times; return to oxygen from nitrogen gas, on the other hand, required 2½ to 3 minutes for the spike to first appear and longer still for it to approach normal amplitude. It is significant that the changes in spike height and conduction velocity in nitrogen are such as to be expected from an excess of potassium (31, 32).

The restoration of both the resting potential

Table 7 demonstrates the behavior of nerves first in oxygen then in nitrogen after one-hour equilibration in Ringer or in a low sodium Ringer. The potassium losses during anoxia are about the same in either Ringer—perhaps somewhat less in the low sodium Ringer if allowance is made for the initial tendency of potassium loss in this solution to be maintained at a somewhat higher level than in the control. Sodium liberation, which is chiefly or exclusively from the extracellular spaces (33), occurs at a continually decreasing rate in accord with the exponential function observed in controls and with no indication of an additional decrease when nitrogen is introduced. Experiments with longer equilibration periods in regular and low sodium Ringer were also tried but were unsatisfactory because potassium losses in oxygen were very high in the latter solution; respiratory inhibition probably

is involved (34). The experimental results therefore demonstrate that potassium release in nitrogen is not contingent upon sodium entry; from this it may be concluded that potassium exit is the primary consequence of metabolic inhibition and sodium influx is secondary. In the absence of analytical data, the same relationships may be assumed to apply to potassium reabsorption during recovery.

Kinetics. THE RESTING STATE. From the available facts we may conclude that a nerve in apparent equilibrium with its environment with respect to potassium and sodium achieves this by the absorption of potassium at a rate exactly equal to the tendency of this ion to escape. Evidence has been presented (8, 10, 11) for regarding the enzymatic process of uptake to be directly related to the potassium concentration at the fiber surface, K_s; as a first approximation the relation may be considered linear. The rate of escape would be governed by physical characteristics of the system such as permeability and concentration differences. The rate of increase of the intracellular concentration, K_i, due to metabolism is therefore

$$\frac{dK_i}{dt} = kK_s, \qquad (1)$$

and the rate of decrease because of physical factors is

$$\frac{dK_i}{dt} = -P_1(K_i - K_s); \qquad (2)$$

in the steady state these are equal and opposite, hence, since $K_s \ll K_i$,

$$\frac{k}{P_1} = \frac{K_i}{K_s}. \qquad (3)$$

The rate constant, k, for metabolism should therefore be greater than the permeability constant P_1 (which also includes geometrical factors) by the ratio of intracellular to extracellular potassium concentrations. On the basis of the potassium-potential relationship, an approximate indication of the relative magnitudes of these constants may be obtained from the maximal rates of post-anoxic repolarization and of anoxic depolarization. These are of the order of 100:1 in frog nerve, which is roughly the potassium ratio in this tissue (see below).

Equation 3 is of importance in indicating that alterations in metabolism as well as of the permeability will change the equilibrium. If valid it provides one means of evaluating permeability changes which may be induced by experimental agents like cocaine that leave respiration unaltered. This is presently under investigation.

ANOXIA. The washing and anodal repolarization experiments previously mentioned indicate that structural characteristics related to the functional activity of nerve remain intact for long periods during anoxia. Glycolysis serves as a source of energy, but only to a limited extent; according to Feng (35) lactic acid production accounts for the residual energy release of frog nerve during anoxia. The combination of anoxia and iodoacetate therefore may be regarded to achieve practically complete cessation of the metabolic machinery for potassium uptake. Let us assume that structural integrity is still maintained.[1] Figure 3 gives the simplified model of a nerve and the differential equations derived there-

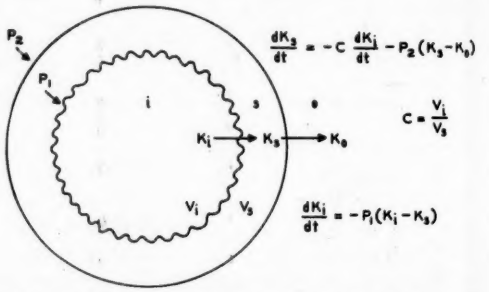

Fig. 3. SIMPLIFIED MODEL of a nerve in cross-section and the equations assumed to underly potassium transfer in the absence of metabolism.

from for potassium diffusion into a stirred medium, o. The individual fibers are considered to constitute a single giant fiber of volume V_i bounded by a membrane whose physical characteristics are described by the constant P_1.[2] Surrounding this is the lumped extracellular space, of volume V_s, bounded in turn by a surface which in frog nerve is represented chiefly by the epineurium and by the constant P_2.[2] That the epineurium interferes with ionic movement was shown many years ago by the direct analytical data of Fenn *et al.* (33): chloride and most of the sodium (which few will deny exist largely in the extracellular space) diffuse from frog nerve into an electrolyte free medium with an exponential time course. When the chloride data

[1] Functional recovery was noted after 12 hours under these conditions and after 24 hours in Ringer.

[2] $P_1 = h_1A_1/V_i$, $P_2 = h_2A_2/V_s$, where the h's are the customary permeability coefficients.

are plotted semilogarithmically a straight line with a time constant of 200 minutes is obtained; thus a single diffusion process is indicated and P_2 under their conditions is found to be 0.5×10^{-2} min.$^{-1}$. Our own preliminary determinations of P_2 from sodium measurements are closer to 10^{-2} min.$^{-1}$; the larger value may be due to more effective stirring. The slowness of potassium escape during anaerobiosis, even when the nerves are desheathed, indicates that P_1 is substantially smaller and that the rate of anoxic loss provides a rough estimate of this constant. It may be taken as $\frac{1}{3}$ P_2 for frog nerve. Additional data required are the intracellular concentrations of the cations and the volumes of the intra- and extracellular spaces. The shrinkage of frog nerve in Ringer made hypertonic with sodium chloride

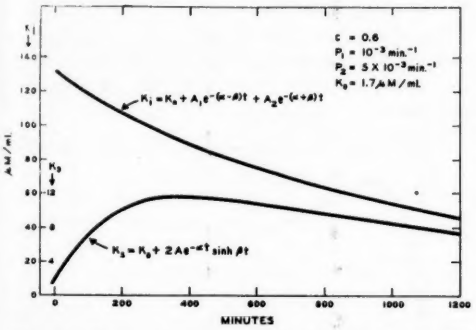

Fig. 4. TIME COURSE of the intracellular (K_i) and extracellular (K_s) potassium concentrations derived from the equations in fig. 3 and with the constants for frog nerve.

(36) agrees with available chloride data (33) in assigning (in round numbers) the volumes given in table 8. From these and whole nerve analyses the axoplasmic concentrations shown have been computed. Estimates for corresponding data in crab nerve are also given.

Figure 4 illustrates the general forms of the solutions of the equations in figure 3.[3] The specific curves shown were derived by assigning the experimentally available values for frog nerve to the constants shown. The surface potassium concentration K_s goes through a maximum while the intracellular concentration K_i declines according to a somewhat less than exponential time function.

Figure 5 illustrates the time course of polarization change when the membrane potential is assumed to obey the indicated relation for a concentration cell with shunt. On a logarthmic time scale an initial 'slow phase' is followed by a nearly linear decline, which in turn is succeeded by a 'slow phase' at very late times; this is exactly as found by Lorente de Nó (16) who conducted his experiments with the nerves in contact with solution. The linear time scale provides a more correct representation of the actual rates involved and is in keeping with time curves indicated by earlier experiments (15).

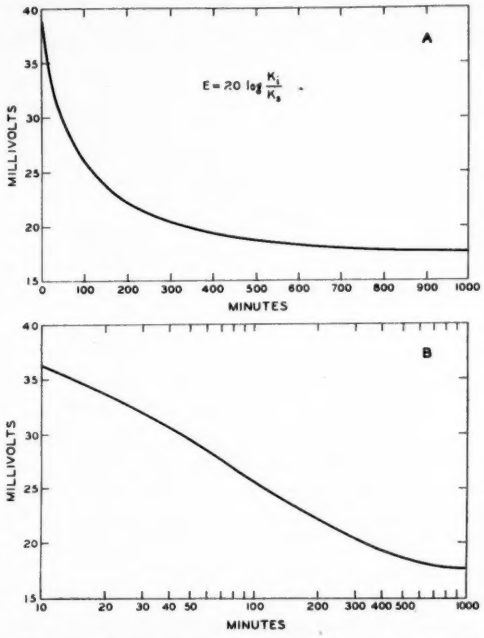

Fig. 5. TIME COURSE of membrane potential change as obtained by the introduction of the theoretical values for K_i and K_s into the equation shown.

The analysis reveals that it is erroneous to consider the very late slow depolarization as an approach to ionic equilibrium. Figure 6 summarizes data collected over a long period to test the validity of this analysis and compares them with a theoretical curve derived from the constants shown. The theory describes potassium release for the entire 24-hour period when iodoacetate is present; in Ringer there is an initial deviation, apparently because of glycolysis, but thereafter the theoretical time course is followed. It seems necessary to conclude that the physical restraints to potassium diffusion are essentially unchanged over these very long periods as originally assumed. Moreover, another procedure is at hand for evaluating changes in permeability

[3] The author is especially indebted to Dr. Jacob Lieberman for a description of a method of solution.

from alterations in the rate of potassium escape; it may also serve to distinguish between metabolic and permeability effects by experimental agents.

POST-ANOXIC RECOVERY. A description of events in this case is more complex. The time constant for metabolism, i.e. 1/k, is small, which implies rapid potassium uptake; hence boundaries which were negligible during anoxia must be considered for an analysis of recovery. For example, the depolarization effects of applied potassium are still not instantaneous in desheathed nerve (35) but occur at a rate suggesting a time

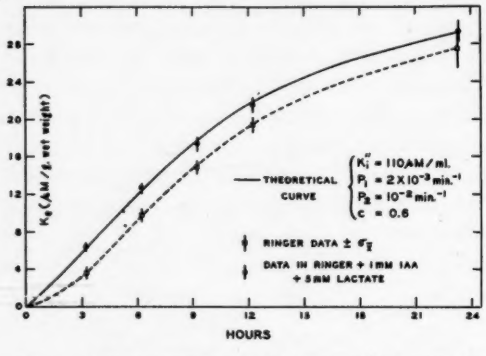
K_o (AM/g. wet weight)
HOURS
THEORETICAL CURVE
$K_i'' = 110 AM/ml.$
$P_1 = 2 \times 10^{-3}$ min.$^{-1}$
$P_2 = 10^{-2}$ min.$^{-1}$
c = 0.6
RINGER DATA ± $\sigma_{\bar{x}}$
DATA IN RINGER + 1mM IAA + 5mM LACTATE

Fig. 6. COMPARISON of the theoretical relation, derived from the equations in figs. 3 and 4, with experimentally obtained data for the net potassium release by frog nerve in nitrogen when iodoacetate (IAA) is present or absent.

constant of 10 minutes, or P = 0.1 min.$^{-1}$ The relative volumes of the compartments into which such boundaries divide the extracellular space also must be known; the difference in the osmotic response of frog nerve to Ringer made hypertonic with sucrose and sodium chloride (36) may be assumed to indicate two such compartments and to provide their volumes, as given in figure 7.[4] The lowered intracellular potassium concentration probably contributes to an elevated respiratory level following anoxia[5] and consequently must be taken into account in a description of events. For mathematical simplicity, the nerve may be assumed to have been mounted in gas during anoxia and upon return to oxygen; the equations in figure 7 then suffice to describe the relationships. These lead to a third order differential equation, the solution of which for potassium at the fiber surface, K_a, is given in figure 8. The variations in the concentration possess the rapidity, the overshooting, and subsequent decline to be expected from the corresponding electrical behavior (7, 15). The chief value of these calculations at present lies in the demonstration that essential

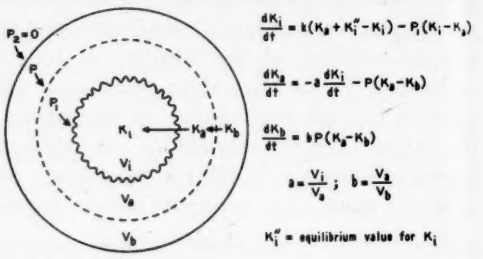

Fig. 7. SIMPLIFIED MODEL of a gas-mounted nerve in cross-section and the equations assumed to underly potassium transfer during post-anoxic recovery.

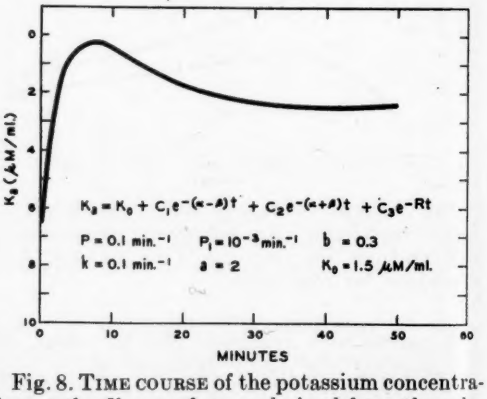

Fig. 8. TIME COURSE of the potassium concentration at the fiber surface as derived from the relations in fig. 7 and with the constants for frog nerve.

features of post-anoxic repolarization are describable from known or measurable properties of nerve. An interesting feature of the derivation is that it is oscillatory. A concrete basis is therefore provided for examining the possibility that more rapid oscillatory bioelectrical phenomena, such as are observable in single fibers, are also due to the interplay of physical and metabolic factors; in this case the sheaths surrounding the individual axons may have an important part because of the rapidity of ionic shifts.

Energetics. The encouraging results obtained in the above attempts to describe nerve behavior in terms of the ionic shifts seem to justify a

[4] Gradients set up around individual fibers as a consequence of the diffusion restrictions by connective tissue may be a closer representation of the situation; the present assumptions may be regarded as a convenient rough equivalent.

[5] Conditions leading to a depletion of intracellular potassium (e.g. anoxia, calcium precipitants, or veratrine) augment respiration (37, 38, 24).

preliminary examination of some aspects of the energetics from the same standpoint. The computations to be described serve to indicate limitations of available data and to check the validity of the original assumptions.

In ionic exchange, where electrical terms cancel, the free energy change or the work done in the transfer of n mols of one of the ion species between two phases at concentrations C_i and C_s is given by

$$\Delta F = nRT \ln \frac{C_i}{C_s} \qquad (4)$$

when *a*) the concentrations are the same as the activities and remain unaltered and *b*) the standard chemical potentials in the two phases are the same. Should one or both concentrations change appreciably—as they must under prolonged experimental conditions, particularly when the extracellular fluid volume is limited—then account must be taken of the dependence of C and hence of the chemical potentials on n. The total free energy change will be the sum of the free energy changes for each ion.

THE RESTING STATE. According to the postulates underlying the kinetics, the cation redistribution when the metabolic machinery is turned off is a measure of the rate of ion transfer by metabolism during the steady state. The rate of work (or the 'free energy flux') is obtained by differentiating *equation 4* with respect to time:

$$\Delta \dot{F} = \frac{d(\Delta F)}{dt} = RT \ln \frac{C_i}{C_s} \frac{dn}{dt} \qquad (5)$$

No assumptions other than those inherent in *equation 4* are involved. Less general considerations, discussed previously in this symposium, lead to the same relation. The formula therefore employs the steady state concentrations (table 8) and the rate of ion transfer provided by media analyses corrected for potassium retained in the extracellular space.

In table 9 the figures obtained[6] are compared with available heat data or with the caloric equivalents based on respiratory measurements (39). In the case of frog nerve, where the lower members of the glycolytic cycle are a major source of energy, oxidation is found to yield a free energy change exactly equal to the thermal values (ca. 0.11 cal./μ M O_2 consumed, as calculated from the data in reference 40). In this particular instance the heat figures suffice for the calculation of efficiency. Because of the sparsity of information on the specific reactions involved in the other cases to be considered, heat output

TABLE 8. COMPONENTS OF CRAB AND FROG NERVE ESTIMATED FOR 1 GM. WET WEIGHT TISSUE AT REST

SPECIES	DRY WT.	V_s[1]	V_i[2]	K_o[3]	K_i[4]	Na_o[3]	Na_i[4]
	mg/gm.	*ml/gm.*	*ml/gm.*	*μM/ml.*	*μM/ml.*	*μM/ml.*	*μM/ml.*
L. emarginata	100	0.25	0.65	10.4	310	520	80
R. pipiens	200	0.50	0.30	1.7	131	110	32

[1] Volume of extracellular space. [2] Volume of intracellular space. [3] Concentrations in the medium. [4] Concentrations in the axoplasm.

TABLE 9. RATES OF POTASSIUM AND SODIUM SHIFTS IN NITROGEN (CORRECTED FOR RETENTION IN EXTRACELLULAR SPACE), COMPUTED MINIMUM RATES OF WORK, AND RELATED EXPERIMENTAL DATA ON RESTING HEAT PRODUCTION

SPECIES	K	Na	$\Delta\dot{F}_K$	$\Delta\dot{F}_{Na}$	$\Delta\dot{F}_{Tot}$	HEAT RELEASE	EFFICIENCY
	μM/gm.hr.	*μM/gm.hr.*	*cal/gm.hr.*	*cal/gm.hr.*	*cal/gm.hr.*	*cal/gm.hr.*	%
R. pipiens[1]	3.4	4.7	8.8×10^{-3}	3.4×10^{-3}	1.2×10^{-2}	$8\text{–}28 \times 10^{-2}$[2]	4–13
L. emarginata[3]	26	26[4]	53×10^{-3}	29×10^{-3}	8×10^{-2}	69×10^{-2}	10

Heat data from Feng (39).
[1] IAA present. [2] Based on the range of respiratory data (39). [3] No IAA present. [4] Assumed.

[6] Where sodium data are lacking, sodium movement has been assumed equal and opposite to that of potassium. When both ions are followed, somewhat more sodium usually is found to be involved, hence another ion probably is undergoing transfer. It may be anticipated, therefore, that some error is present even where sodium data are available.

will be assumed to equal the total free energy change for the efficiency computations.

Within the limitations of the data, the efficiency is about the same for frog and crab nerve, viz., 10 per cent, when the energy turnover in the steady state is assumed to be concerned exclusively with ion transfer. Allowance for the contributions of processes concerned with other than ion distribution to energy liberation may make these efficiency figures higher.

STIMULATION. A satisfactory evaluation of the energy relationships of activity involves correction not only for potassium retention in the extracellular space but for potassium reabsorption during the stimulation period as well. The use of inhibitors selective for 'activity metabolism' (41) may serve to minimize the second complicating factor. The available data must presently be regarded on the low side. In the case of crab nerve this error is probably small because of the slow recovery process.

Table 10 gives available thermal (39) and calculated figures for the single impulse. In frog nerve the initial heat is of the same magnitude as the free energy decrease, but this may be regarded a coincidence since in an ideal solution no heat would be evolved as a result of mixing. Some heat transfer may be anticipated from the non-ideality of the system but, unless other than simple dilutions are involved within the fiber, this is probably small. It is noteworthy that the decline in initial heat with continued repetitive stimulation or with increased frequencies of stimulation (39) is in the direction to be anticipated from the changes in concentration and in the associated activity coefficients.[7]

Again assuming that thermal data (i.e. the recovery heats) measure the total free energy change during recovery, we obtain an efficiency of about 45 per cent for crab nerve and of 5 per cent for frog nerve. The high value for invertebrate tissue indicates that the restoration of the normal ion balance is a major concern of recovery; the involvement of some recovery reactions in other processes would lead to a prohibitively high efficiency. It is hardly likely that the use of heat rather than free energy data in the calculations is in itself responsible for this high value. The low figure for frog nerve cannot be taken seriously until an estimate is available of the potassium failing to appear in the medium during stimulation because of active reabsorption; a high absorption rate would give a spuriously low efficiency.

These considerations do not imply a direct dependence of impulse transmission on recovery processes. This would be contrary to the conclusion reached earlier from the washing and recharging experiments. Rather, recovery may be regarded as concerned *a*) with minimizing the extracellular accumulation of potassium and *b*) with preserving an adequate reserve of intracellular potassium for impulse production. On this basis the interference with recovery respiration achieved recently with azide (41) need not be expected to impair conduction as long as the potassium levels within and without the fibers are not altered excessively; but, as in the case of inhibitors less specific for 'activity metabolism,' azide may be expected to hasten the onset of failure during continual activity.

TABLE 10. POTASSIUM AND ASSUMED SODIUM SHIFTS FOR A SINGLE IMPULSE (CORRECTED FOR RETENTION IN THE EXTRACELLULAR SPACE), CORRESPONDING FREE ENERGY CHANGES, AND AVAILABLE DATA FOR HEAT PRODUCTION

SPECIES	K	Na	ΔF_K	ΔF_{Na}	ΔF_{Tot}	INITIAL HEAT	RECOVERY HEAT
	μM/gm. impulse		*μcal/gm. impulse*			*μcal/gm. impulse*	
L. emargi-nata	5.3×10^{-3}	5.3×10^{-3}	10.7	5.9	16.6	0.73	36
R. pipiens	2.9×10^{-5}	2.9×10^{-5}	7.5×10^{-2}	2.2×10^{-2}	9.7×10^{-2}	6.7^{1}–$26^{2} \times 10^{-2}$	2

[1] At 20°C. [2] At 0°C.

[7] Dr. Hearon very kindly provided the theoretical basis for these remarks on initial heat.

NOTE ADDED IN PROOF

A series of publications by Feng and his associates in the 1950 issue of the *Chinese Journal of Physiology*, received subsequent to the submission of this manuscript, should be noted; of particular interest is the paper (*Chinese J. Physiol.* 17: 247, 1950) corroborating the correlation be-

tween potassium release and depolarization indicated by our early indirect experiments and discussed with Dr. Feng prior to publication (7, 8). Limitations of space have compelled a scrupulous neglect of recent pertinent data on muscle; however, attention must be called to the work of Fleckenstein and his associates, recently summarized in detail (*Arch. Exper. Path. Pharmakol.* 212: 54, 1950). Their results are of particular importance in indicating significant relationships between electrochemical changes and muscle shortening under conditions similar to those which have been described for nerve.

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MACHINE EFFICIENCY OF ASSIMILATIVE PROCESSES

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THE EFFICIENCIES with which living organisms convert food materials to protoplasmic constituents or energy stores during growth, repair, or maintenance are of economic importance. They also carry important theoretical implications for students of intermediary metabolism. Investigations dealing with this problem have been conducted using organisms ranging from mammals to microorganisms. (Phototrophic nutrition poses special problems and will be neglected here.) The purpose of this paper is to analyze some of the results obtained in an attempt to estimate the efficiency with which such conversions can or do occur. The varied and frequently arbitrary conventions used by different workers will be examined, and the doubts and difficulties which beset students of the problem will be emphasized. Conventions which make possible the application of thermodynamic as well as thermochemical analysis to studies of heterotrophic nutrition are introduced. It is hoped that these will encourage further investigation and thermodynamic analysis particularly in mammalian nutrition.

The discussion which follows is divided into five parts: *1*) Definition of the machine efficiency of assimilation; *2*) problems connected with ascertaining this efficiency; *3*) efficiencies reported or calculated here for microorganisms; *4*) factors affecting assimilation efficiency; and *5*) calculation of a machine efficiency of assimilation in the rat.

The literature cited is intended to be illustrative rather than comprehensive and it is left to the reader to consult original papers for details of methods and results. All free energy calculations are based on values to be found in previous publications (1, 2).

1. DEFINITION OF MACHINE EFFICIENCY OF ASSIMILATION

Two widely differing measures of assimilation efficiency are in common use. Thus, in mammalian nutrition studies, efficiency is ordinarily calculated (3) as 100 × calories stored ÷ assimilable calories ingested. In studies on microorganisms (1, 4, 5) efficiency has been calculated as 100 × the free energy cost of assimilative reactions ÷ free energy available from catabolic reactions. An efficiency calculated in either way is commonly called the 'efficiency of growth.' Growth in the complete sense involves cell division which requires energy (movements of cytokinesis and karyokinesis, creation of new surface, etc.) and organization with an unknown entropic contribution. Most of the studies referred to below, however, involve simply the accretion of protoplasm or of stored materials such as glycogen or fat. I shall therefore speak of efficiencies of assimilation rather than of growth.

The two different customs referred to above represent basically different approaches to the problem of assimilation. On the one hand, the calories in corn conserved by a cow are of economic interest. On the other, the ratio of free energy required to that made available for assimilation may either support or embarrass particular ideas about intermediary metabolism and the mechanisms whereby energy is trapped and utilized. I shall refer to efficiencies calculated in the first way as 'caloric conservation efficiencies' or to the 'caloric conservation coefficient.' For the quantity 100 × free energy cost of synthesis ÷ free energy available from catabolism I shall use the term 'machine efficiency of assimilation' (6).

By free energy available from catabolism I shall mean that over and above a basal level[2] observed in nonassimilating control organisms. In microorganisms this 'basal' level is ordinarily a fasting level (1, 5). In mammals, as we shall see, the maintenance level of metabolism serves

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[2] Subtraction of the endogenous metabolism of microorganisms may be questionable in other connections (7). For the present purpose, failure to do so would imply that the cost of other processes during assimilation is zero. In many cases the correction is inconsequential, particularly if the endogenous metabolism is a small fraction of the exogenous.

best as the base (8). Subtraction of a maintenance metabolism in microorganisms would be preferable, but this is difficult to establish. The fact that metabolism during assimilation in microorganisms is many times (5- to 40-fold) greater than the endogenous rate minimizes the error involved.

2. POSSIBILITY OF ESTABLISHING MACHINE EFFICIENCIES OF ASSIMILATION

At first glance, calculation of the efficiency with which free energy available from catabolism is captured for purposes of assimilation appears simple. Assimilation in principle consists of the chemical conversion of one or more compounds to some other compound(s). Given the free energies of formation ($\Delta F_{form.}$) of reactants and products, their activities, and the number of mols converted, the free energy required is calculable. The same being true for catabolic reactions, the energy available and thence the machine efficiency of assimilation emerge. A closer inspection of the details involved, however, forces a more pessimistic outlook.

The most glaring present-day deficiency in the knowledge needed for this purpose is our complete ignorance of $\Delta F_{form.}$ values for proteins. Indeed the values for many amino acids are unknown, and are not calculable by usual procedures (9). Entropy values associated with structural improbabilities in proteins further complicate the matter. It is no wonder therefore that those who have attempted to estimate assimilation efficiencies have either eliminated protein synthesis by omitting a nitrogen source (2, 5) or have made arbitrary assumptions regarding the energy cost of protein synthesis (1, 4).

The above difficulties regarding the cost of protein synthesis could probably be removed by appropriate experimental work. Heat-capacity measurements at low temperatures on a few amino acids and a few crystalline proteins would permit calculations regarding entropies from the third law of thermodynamics. There would still remain certain difficulties not so easily overcome. These concern carbohydrate and fat formation as well as protein formation.

What shall we say is the work done when an animal converts a food, say glucose, to fat? The overall process is exergonic. Thus for glucose to triolein assuming passage through a C_2 stage with the necessary generation of 2 CO_2:

$$\underset{(0.005\text{M})}{C_6H_{12}O_6} + \underset{(0.2\text{ atm.})}{0.385\ O_2} \rightarrow \underset{(\text{liq.})}{\tfrac{1}{57}(C_{57}H_{104}O_6)} + \underset{(0.055\text{ atm.})}{2\,CO_2} + \underset{(\text{liq.})}{2.35\,H_2O}$$

$$\Delta F_{310} = -111{,}671 \text{ cal.} \quad (1)$$

If we accept this at its face value, no work is done and the efficiency of assimilation is zero or indeterminate. This situation is not confined to mammalian nutrition or to foods like glucose. Thus Blum *et al.* (2) were faced with the problem of assimilation of ethanol with starch being synthesized. Again the overall reaction under their conditions was exergonic.

$$\underset{(10^{-4}\text{M})}{C_2H_5OH} + \underset{(0.2\text{ atm.})}{O_2} \rightarrow \underset{(\text{sat. soln.})}{\tfrac{1}{3}(C_6H_{10}O_5\cdot\tfrac{2}{24}H_2O)} + \underset{(\text{liq.})}{\tfrac{7}{2}H_2O}$$

$$\Delta F_{298} = -78{,}720 \text{ cal.} \quad (2)$$

The escape from the above dilemma proposed (2) is something more than a convention. Invoking the known intermediary metabolic steps and the fact experimentally demonstrable in their experiments that acetic acid appears at least transitorily in the conversion of ethanol to carbohydrate the authors reasoned as follows: Energy is released in the oxidation of ethanol to acetate; some further energy is released by the complete combustion of a fraction of the acetate; to convert the remainder of the acetate to amylose is an endergonic process. Taking the work done as that of converting the acetate to amylose, and the energy available as the sum of the two oxidative processes, these authors found an efficiency essentially the same as that found for acetate oxidation and assimilation in *Chilomonas*.

The above line of reasoning can be applied to the glucose-to-fat conversion cited above. Thus glucose in all probability is first converted to two C_2 fragments + 2 CO_2 + water. To synthesize fat from these C_2 fragments is an endergonic process. A more detailed analysis of this situation is given in *section 5*. The important point to be recognized is this: Whenever in an overall exergonic process, a point is reached at which the remaining steps are endergonic, the problem for the biologist is to ascertain how efficiently the free energy yielded in the exergonic steps is captured to drive the endergonic ones. This is no violation of the principle that the overall free energy change is independent of the path (10). This must and will still be true. However, one does not have to be frightened into ignoring everything except the overall ΔF. Common sense,

if nothing else, tells us that if endergonic steps occur, they must be driven. Other steps in the same overall process are candidates for this function on an equal basis with steps in parallel reactions.

The above convention introduces certain difficulties not encountered in simpler situations. Thus in the ethanol-starch conversion, the concentration of the intermediary acetate affects the actual ΔF values of both the exergonic and endergonic reactions. In the glucose-to-fat conversion the identity and concentration of the C_2 fragment are of critical importance. These however are transient difficulties to be overcome by future studies of intermediary metabolism and quantitative analyses of the cells or fluids concerned.

Finally we may consider the methods available for establishing the percentage of available foodstuff degraded and the percentage assimilated. These methods differ in detail but are in essence the same whether the assimilating organism be a bacterium or a mammal. Three mutually supporting methods are available:

1) Direct chemical analysis. This is most easily practiced with mass cultures of microorganisms from which aliquots can be withdrawn from time to time. It is the least equivocal of the methods and knowledge of the details regarding conversions occurring is limited only by existing analytical methods and the experimenter's patience.

2) Calorimetry. By measuring the extra heat produced by an organism presented with a limited and known amount of substrate, the degradation-assimilation ratio can be calculated under certain circumstances (2, 5). Ordinarily chemical analyses or respiration measurements must be conducted to confirm the assumptions regarding the reactions responsible for generation of the heat.

3) Respirometry. The extra O_2 consumed and/or CO_2 produced can be used in place of heat production as above. Again auxiliary information from direct analyses or heat measurements may be required.

The practical difficulties met with in calorimetry and respirometry for the above purposes are well illustrated in figure 1 taken from an experiment carried out in my laboratory (11). This figure might just as well represent heat or CO_2 production as O_2 consumption.

It is a well-established fact that in such experiments the extra O_2, CO_2 or heat is less than that theoretically derivable from the added substrate (2, 5, 7). It is commonly assumed that when a sharp break occurs in the upper curve the added substrate has been exhausted through combined degradation and assimilation. With glucose as substrate this has been demonstrated analytically (5). With other substrates it seems the only explanation compatible with other known facts (7, 12). It would be comforting, however, to have direct analyses for other substrates at the time of this sharp break in the curve.

The difficulties inherent in this method of establishing oxidation-assimilation ratios stem from the following:

1) The endogenous rate of O_2 consumption falls off with time. In yeast this decline is first order (5). In *Chilomonas* there is usually an abrupt change in rate after 2 to 3 hours (*lower curve*, fig. 1).

2) The rate of O_2 consumption following the exhaustion of added metabolite exceeds even the original endogenous rate for some time. This excess in rate after the break is roughly proportional to the amount of substrate assimilated (7, 11).

The net effect of the above is to indicate a progressively greater percentage of substrate catabolized the longer one waits to assess the gas or heat increment. In my opinion the best choice one can make is ΔY_1 (fig. 1). To take the increment at any later time probably includes metabolism of material recently assimilated. This problem has long plagued experimenters in this field, but it has become particularly important in some of our recent studies.

So long as a large fraction of added substrate is oxidized and the calculated efficiency is low it carries no embarrassing theoretical implications. However, in experiments on *Chilomonas* metabolizing acetate we have recently recorded oxidation-assimilation ratios near 1:4. With starch as the end product this implies an efficiency near 50 per cent under our conditions. Furthermore, the energy captured is approaching the maximum which can be accounted for by high energy phosphate bond ($\sim$P) formation. Should the apparent efficiencies increase by much, the number and/or energy value of $\sim$P bonds formed would have to be increased, other mechanisms of energy capture would have to be found,

or our assumptions regarding the cost of synthesis would have to be revised.

To summarize, in preparation for the next section: So long as the end product of synthesis is carbohydrate or fat, quite accurate calculations of the efficiency of assimilation frequently can be made. When protein is being synthesized no exact calculations are possible. Often the overall reaction of assimilation is exergonic. Only by splitting it into its exergonic and endergonic components can any calculations of efficiency be made. With these reservations I present the data on microorganisms below.

3. BEST PRESENT ESTIMATES OF ASSIMILATION EFFICIENCIES

The most precise calculations of the efficiency with which assimilation occurs have been made using data obtained with microorganisms (1, 2, 4, 5). In a pioneering effort Baas-Becking and Parks (4) in 1927 analyzed the data of many workers who had studied the growth of autotrophic bacteria. The data available to them gave mols of CO_2 fixed per mol of inorganic energy yielding material oxidized. Defining efficiency of growth as I have defined machine efficiency of assimilation they obtained values near 6 per cent for most forms, hydrogen bacteria being exceptional with an efficiency of 26 per cent.[3] Until the recent studies of Blum *et al.* (2) on *Chilomonas* the figures for hydrogen bacteria were the highest ever reported.

In making their calculations Baas-Becking and Parks (4) made the simplifying assumption that all of the CO_2 fixed was converted to solid anhydrous glucose. The equation used by them was

$$\underset{(0.0003\ \text{atm.})}{CO_2} + \underset{(\text{liq.})}{H_2O} \rightarrow \underset{(\text{solid})}{\tfrac{1}{6}(C_6H_{12}O_6)} + \underset{(0.2\ \text{atm.})}{O_2}$$

$$\Delta F_{298} = +118{,}000 \text{ cal.} \quad (3)$$

With CO_2 as a carbon source this simplifying assumption leads to a probably negligible error in view of other uncertainties. Thus, if the amylose hexose unit of glycogen or starch (1) is taken as the product in the above equation $\Delta F_{298} = +118{,}862$ cal.

For that fraction of the CO_2 converted to fat, using triolein as typical

$$\underset{(0.0003\ \text{atm.})}{CO_2} + \underset{(\text{liq.})}{\tfrac{53}{57}H_2O} \rightarrow \underset{(\text{liq.})}{\tfrac{1}{57}(C_{57}H_{104}O_6)} + \underset{(0.2\ \text{atm.})}{\tfrac{80}{57}O_2}$$

$$\Delta F_{298} = +147{,}587 \text{ cal.} \quad (4)$$

[3] See also Burk (6).

In the absence of free energy values for protein formation we can do no more than state the value of $\Delta H = +126{,}000$ cal. per mol of CO_2 converted to egg albumin based on a heat of combustion of 5.7 cal/gm. The efficiencies calculated (4) for autotrophic bacteria would therefore be increased to no more than 7 to 8 per cent for most forms if closer account were taken of the end products.

When we turn to heterotrophic nutrition, the assumption that solid anhydrous glucose is the end product of synthesis can no longer be employed without introducing an intolerable error.

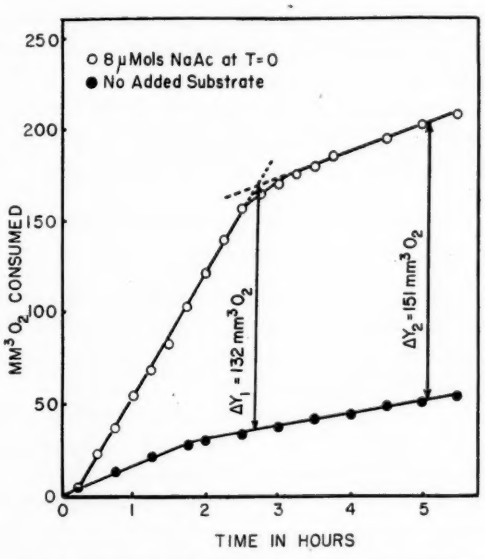

Fig. 1. TIME COURSE of O_2 consumption following addition of 8 μmols Na acetate to suspension of *Chilomonas* at pH 6.0. Temperature 25°C., 4.0 ml. cell suspension 3.5 x 10^5 cells/ml.

Thus, if 5 per cent glucose (0.28 M) itself is the substrate

$$\underset{(0.28\text{M})}{C_6H_{12}O_6 \cdot H_2O} \rightarrow \underset{(\text{solid})}{C_6H_{12}O_6} + \underset{(\text{liq.})}{H_2O}$$

$$\Delta F_{298} = +1885 \text{ cal.} \quad (5)$$

On the other hand, taking the actual final product as amylose hexose units in saturated solution yields

$$\underset{(0.28\text{M})}{C_6H_{12}O_6 \cdot H_2O} \rightarrow \underset{(\text{sat. soln.})}{C_6H_{10}O_5 \cdot \tfrac{25}{24}H_2O} + \underset{(\text{liq.})}{\tfrac{33}{24}H_2O}$$

$$\Delta F_{298} = +3878 \text{ cal.} \quad (6)$$

Large errors (a factor of 2–3) in estimating the

work done can easily be introduced in this way. In all of the studies summarized in table 1 adequate account was taken of the final product of synthesis. In making calculations from Barker's (7) data I have accepted his conclusion that only carbohydrate was formed and used the amylose hexose unit as the final product taking as its free energy of formation −217,851 cal. (1). For lack of space I have dealt only with Barker's data involving acetate and butyrate. Data presented by Wilson and Peterson (13) and Fulmer (14) could also serve as the basis for interesting calculations but assumptions used would have to be explicitly stated.

It will be seen from table 1 that reported values for the efficiencies of assimilation of organic substrate by heterotrophic forms fall in the same range as those reported for autotrophic bacteria (4). Whether these are the highest possible efficiencies will be discussed below.

4. FACTORS AFFECTING ASSIMILATION EFFICIENCY

The natural tendency of investigators studying the efficiency with which substrates are assimilated has been to employ temperatures, pH values, salt concentrations, etc., optimal in the sense that they produce the most rapid growth of the organism. The carbon and energy source has frequently been below optimal in the above sense. There is no *a priori* reason to assume that conditions which lead to most rapid cell division or increase in mass of protoplasm also lead to the most *efficient* assimilation. Indeed there is evidence to the contrary.

Brücke (15) studying anaerobic assimilation of glucose by yeast found that monoiodoacetate (IAA) at a concentration which inhibited fermentation by 86 per cent reduced conversion of glucose to glycogen by only 57 per cent. The experiments were of such duration as to preclude any explanation based on temporary nonsteady state phenomena. The improved assimilation-fermentation ratio raised the efficiency of the process from ca 2 per cent to ca 6 per cent. A similar effect of fluoride on assimilation of glucose by yeast has been reported (16). These findings may be interpreted as follows: The fermentation of glucose is inhibited by IAA and fluoride while the assimilative reactions from glucose to glycogen are unaffected. Any existing excess of energy production over that actually required for synthesis would thus be reduced.

The converse of the above situation is seen in the effect of such inhibitors as IAA and fluoride in reducing the percentage assimilation of substrates such as acetate and butyrate (12). Here the assimilative pathway is blocked while energy release is essentially unaffected. Cyanide and azide reduce assimilation of substrates but their effect is not easily explained. Dinitrophenol in appropriate concentrations leads to complete oxidation of acetate in yeast (5) and *Pseudomonas* (12), i.e. zero assimilation efficiency. Presumably this results from interference with oxidative generation of ~P bonds (17).

The report of Blum *et al.* (2) that the oxidation-assimilation ratio for acetate is greater in the presence than in the absence of a nitrogen source cannot at present be interpreted. Protein synthesis may require more energy than carbohydrate or fat synthesis. On the other hand it may be less efficiently accomplished. In any event the finding serves as a warning against accepting assimilation efficiencies obtained in the absence of a nitrogen source as a measure of the 'efficiency of growth' (5).

Two factors which might logically be expected to influence assimilation efficiency have been inadequately studied in microorganisms. These are temperature and the nutritional state of the cell. In my own laboratory we have been unable to detect any change in the efficiency of synthesis of starch from acetate by *Chilomonas* over the temperature range from 15° to 30°C. Regarding nutritional state, starving cells for 24 hours has little if any effect on percentage of acetate assimilated. Raising the starch content of the cells by prolonged exposure to acetate in the absence of a nitrogen source (to prevent growth) simply reduces the rate at which acetate is oxidized and assimilated rather than changing efficiency (11).

5. MACHINE EFFICIENCY OF CARBOHYDRATE ASSIMILATION IN THE RAT

As stated earlier it is common practice in studies on mammalian nutrition to calculate the caloric conservation efficiency. If calculations analogous to those given above for microorganisms have appeared, they have escaped my notice. I have attempted some crude and tentative calculations below with three objects in view: First, to stimulate others to make more precise calculations; second, to stimulate the reporting of data which will make such calculations possible; and finally to show that, to a first approxi-

mation, mammals do not differ radically from microorganisms in the efficiency with which they capture free energy for assimilative purposes.

Out of the vast body of knowledge regarding mammalian nutrition relatively few studies are suitable for the present purpose. Measurements of the specific dynamic action of foods measured above the basal (fasting) level must be rejected. Between the basal and maintenance levels of metabolism food apparently serves largely to spare the body stores which otherwise would have to be metabolized. Only above the maintenance level does net synthesis occur. Forbes *et al.* (8, 18) have shown that above the maintenance level, i.e. when net synthesis is occurring, the specific dynamic actions of foods increase sharply. Above the maintenance level the extra heat produced per unit of ingested food is constant over a considerable range (8).

I have chosen as the basis of the computations which follow the data of Kriss *et al.* (19). In these studies rats were first established on a maintenance ration of calves' meal. The average daily heat production was 15,888 cal. When an additional 2.2 gm. of starch having a caloric equivalent of 8228 cal. were fed the mean daily heat production rose to 17,736 cal. The difference of the means was 1848 ca.l with a standard error of 242 cal.

On the basis of the above figures the mean caloric conservation efficiency is 77.5 per cent since only 22.5 per cent of the calories theoretically derivable from the extra fed starch appeared. In order to calculate a machine efficiency of assimilation we must make certain assumptions regarding the end product of the assimilation, the pathway of metabolism, and the conditions under which the conversions occurred.

That the end product stored was chiefly fat there can be little doubt. The rise in R.Q. values produced by the extra starch indicates this, and storage of any appreciable portion of the starch as glycogen would produce an improbable rise in the glycogen content of the tissues. I shall assume therefore that fat was the product of synthesis and use triolein to represent this.

The remaining assumptions which I shall make can be quarrelled with in almost every detail. The absurdities are purposeful, however, in that the pathway and conditions chosen yield what is probably the maximum machine efficiency. Thus I shall assume that the C_2 particle derived from glucose which serves as the starting point for fat synthesis is the acetate ion at a concentration of 10^{-6} molar. Trapping the C_2 particle at an energy level above acetate would decrease the work of synthesis more on a percentage basis than it would decrease the energy made avail-

TABLE 1. MACHINE EFFICIENCIES OF ASSIMILATION OF HETEROTROPHIC MICROORGANISMS

ORGANISM	SUBSTRATE	PRODUCTS	METHODS	EFFICIENCY, %	REFERENCE
Yeast	Acetate	Glycogen	Heat prod.	12.2	3
	Glucose (aerobic)	Glycogen	Heat prod.	2.88	3
	Glucose (anaerobic)	Glycogen	Heat prod.	4.62	3
	Glucose (anaerobic)	Glycogen	CO_2 prod.	ca 4.6	20
Chilomonas	Acetate	Starch	Heat prod.	26.7	8
	Acetate	Starch (and fat?)	Heat prod.	21.9	8
	Acetate	Starch (fat and protein)	Heat prod.	13.1[1]	8
	Acetate	Starch, fat and protein	Direct analysis	17.0[1]	4
	Acetate	Starch	O_2 cons. and CO_2 Prod.	29–46	10
	Ethanol	Starch	Heat prod.	20.2	8
		Starch (and fat?)	Heat prod.	17.1	8
Prototheca	Acetate	Amylose	O_2 and CO_2	16.4	6
	Butyrate	Amylose	O_2 and CO_2	13.8	6

[1] Energy cost of protein taken as that of carbohydrate of equal carbon content.

able for driving the reaction. Other assumptions can be similarly shown to work in this way.

I shall ignore the heat and free energy changes attendant on hydrolysis of the starch, and the hydration, solution and dilution of the derived glucose. Starting then with glucose at a concentration of 90 mg. per cent (0.005 M) I shall write the following overall equation.

$$C_6H_{12}O_6 \cdot H_2O + 1.34\,O_2 \rightarrow$$

$$0.058 \text{ triolein} + 2.68\,CO_2 + 3.97\,H_2O \quad (7)$$

I have calculated $\Delta H_{310} = -176{,}926$ cal. for this reaction. The heat value given by Kriss *et al.* for their starch (3.72 cal/gm.) corresponds to a little more than monohydrated amylose units (M.W. = ca 182). On this basis they fed 0.012 mols/day. The above equation therefore predicts an extra 2100 cal/day. This figure is well within the permissible range of 1364 to 2332 cal. (mean ± 2 S.E.).

To estimate the energy available and required for the conversion I shall break the overall reaction into two components:

$$\underset{(0.005M)}{C_6H_{12}O_6 \cdot H_2O} + \underset{(0.2\text{ atm.})}{1.34\,O_2} \rightarrow$$

$$\underset{(10^{-6}M)}{1.66\,CH_3COO^-} + \underset{(0.055\text{ atm.})}{2.68\,CO_2} + \underset{(pH\ 6.5)}{7.02\,H^+} +$$

$$5.36\,e + \underset{(liq.)}{H_2O} \quad \Delta F_{310} = -264{,}806 \text{ cal.} \quad (8)$$

$$\underset{(10^{-6}M)}{1.66\,CH_3COO^-} + \underset{(pH\ 6.5)}{7.02\,H^+} + 5.36\,e \rightarrow$$

$$\underset{(liq.)}{0.058 \text{ triolein}} + \underset{(liq.)}{2.97\,H_2O}$$

$$\Delta F_{310} = +55{,}072 \text{ cal.} \quad (9)$$

On this basis the machine efficiency is 20.8 per cent. As mentioned above alterations in the adopted scheme to make it more nearly compatible with the realities of intermediary metabolism will tend to lower the efficiency. I can only plead that I would have made such changes if I could. Until the identity of the C_2 fragment, its concentration, and energy of formation are known, a better calculation will be difficult. One point is perhaps worth comment. In classical schemes of representing carbohydrate to fat conversions generation of gaseous O_2 is employed in partial reactions. The scheme I have employed of reserving the H^+-electron pairs for subsequent reductions in fat formation with generation of water is more realistic. It was to achieve balance in this regard without appealing to outside systems that I accepted a slightly high figure for heat production. Trapping the C_2 fragment at a more reduced stage than acetate would allow greater latitude in proportioning oxidation and fat synthesis.

It would thus appear that, employing similar conventions, the machine efficiency of assimilation in a mammal may be quite similar to those observed in microorganisms. Other and more adequate calculations of this type should of course be made. In particular, variations in foodstuff, feeding schedule, and end product synthesized may alter the efficiency. The examples cited in *section 4* bear witness to the fact that the efficiency which does occur may have little relation to the maximum possible efficiency.

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AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

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LOCALIZATION OF SUBSTANCES IN CELLS

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IT IS very gratifying to a cytologist to have an opportunity to address The American Society of Biological Chemists in a Symposium on Histochemistry. Until the later decades of the nineteenth century, cytology and biochemistry were not at all distinct; both were prominent parts of the broad field of physiology. The microscopic structure of tissues always appeared as part of the textbooks, and many of the great physiologists speculated on the possible localization of functions within the structural components of cells. Claude Bernard, for example, suggested that the cell nucleus, the only structure then recognized to be common to all cells, was concerned with synthetic functions—a view that was later brilliantly confirmed by the demonstration that when a single-celled organism is cut in two pieces, only the nucleated half can carry on the syntheses essential for growth. It was under the stimulus of such ideas that in 1869 Miescher devised a method of isolating masses of nuclei from the remainder of the cell, the cytoplasm, and subjected them to chemical analysis. Almost coincident with Miescher's discovery of the very unusual chemical composition of the nucleus, Abbé's greatly improved compound microscope became available—and in the hands of Flemming and others this instrument led to observations of the remarkable nuclear phenomena during cell division and fertilization, which marked the beginning of modern cytology. These early workers by no means regarded themselves as in distinct fields; each was well aware of parallel progress by the other group. Miescher, for example, performed experiments to show that the specific staining of nuclear substance was due to combination of his nuclein with the basic dye methyl green; while it was a cytologist, Altmann, who gave the name nucleic acid to the acidic component of Miescher's nuclein. Beginning early in the twentieth century, however, there developed among both cytologists and biochemists a tendency to work under domination of particular standardized techniques rather than under stimulus of the broad problem of correlation of biological function with intracellular organization. Such specialization eventually led to the almost complete isolation of these two fields, which prevailed until fairly recently. Within the last 10 years happily there has been a reorientation, a return to recognition of the priority of the problem of the significance of intracellular organization. Today there are many cytologists who are anxious to work with biochemists, even to attempt to master some of their techniques; and there appear also to be biochemists who are willing to attempt to localize activities within morphological components like the nucleus, mitochondria, and microsomes.

It is the great charm, the fascination, of study of the microscopic structure of organisms that the shapes of tissue cells and intracellular morphological changes are often so beautifully expressive of the function of the cell. This is well shown by the exocrine glandular cell, such as that of the pancreas, which I shall later use to illustrate the methods of localization of nucleoprotein in cells. This cell is in the form of a pyramid, usually six-sided, with sides in contact with 6 other cells. The base of the pyramid is adjacent to the blood vessels, the apex is in communication with the duct system of the gland which opens into the small intestine. The fine details of cells are usually studied in thin sections, and the probable structures visible in such a thin section are shown schematically in figure 1. The

most conspicuous object is of course the nucleus, within which are scattered masses of chromatin, and a conspicuous nucleolus. The duct end of the cell is largely occupied by a mass of spherical secretory granules, concealed within which is a pair of centrioles, which are organs of cell division. Scattered throughout the cytoplasm are mitochondria, and in the basal region, perhaps also

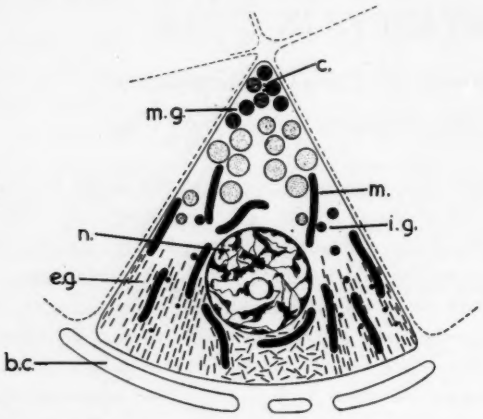

Fig. 1. DIAGRAM of structural features in section of an exocrine glandular cell of the pancreas, after Pollister, 1951 (1). Parts of the boundaries of adjacent cells in the secretory unit, or acinus, are outlined and the blood capillaries near the basal end of the cell are indicated. Abbreviations: *c*, centriole; *e.g.*, basal ergastoplasmic zone, containing submicroscopic microsomes. (The microsomes have not been observed in this type of cell. It is assumed from results on liver cells by Hogeboom, Schneider, and Palade, 1948 (2), that: *a*) the cytoplasmic region of high nucleic acid content must be the site of a major accumulation of microsomes; *b*) the microsomes are asymmetrical, probably thread-like; and *c*) these asymmetrical bodies tend to become oriented parallel with the long axis of the cell, as suggested in Pollister, 1940 (3)); *m.g.*, mature granule, *i.g.*, immature granule; *m*, mitochondrion; *n*, nucleus; and *b.c.*, blood capillary.

elsewhere, there is reason to believe there are rather densely packed sub-microscopic thread-like microsomes. The actual structure is shown in the phase contrast photomicrograph of an unstained one-micron section of a rat's pancreas (fig. 2 *A*). This cell has one principal function, to elaborate the granules which are thrown out into the duct system when the gland secretes the pancreatic juice. This phenomenon clearly suggested that the granules are the precursors of the constituents of the pancreatic juice, for which reason they have long been called *zymogen granules*. As you doubtless all know, this relation to the pancreatic enzymes is supported by the observations of Van Weel and Engel (4), that the peptidase activity of fresh slices of pancreas is directly related to the granule content of the cells. The central problem of intracellular pancreatic function, then, is how in a few hours the cell elaborates a large mass of zymogen granules, which has been estimated to be as much as one-fifth of its own dry weight. Morphologically this process has been well described in beautiful studies, first by Hirsch (5) and later by Duthie (6). In the quite intact gland, drawn out onto a warmed slide through a ventral incision in an anesthetized mouse, they followed the development of individual granules. The granule appears first as a small body in the basal zone, it slowly enlarges and becomes progressively more dense as it moves toward the final position for discharge. There are also marked concomitant changes in the nucleolar part of the nucleus, and in the appearance and staining reactions of the basal part of the cell.

The pancreas is a typical component of a specialized tissue, and like other cells has two categories of visible structures: *a*) the granules which are primarily an expression of the unique cell function, and *b*) the nucleus, mitochondria, centrioles, and probably microsomes which are much like those of all other cells, and which we accordingly are accustomed to consider as the essential cell organelles which are concerned with cell maintenance and other functions which underlie the special tissue activity. These structural features of cells are fairly well worked out, although the picture will surely soon be greatly enriched by developments in electron microscopy. The major unsolved problem is to make this picture meaningful in terms of cellular biology, to determine what specific functions may be attributed to the visible structures in cells. There was a period—not so long ago—when it seemed as if physiologists were satisfied to attribute a majority of fundamental intracellular activities to the properties of a formless sort of soup, which had as its chief asset the heritage of a fine old name—*protoplasm*. I suppose this was patterned after the distribution of hemoglobin in the erythrocyte, which is a most atypical cell indeed. At any rate, there has been in recent years a marked change of viewpoint. As some of the other speakers in this symposium will amplify, it is becoming evident that many important physiological mechanisms are as sharply restricted to

one particular cell component as the mechanisms for gene distribution and photosynthesis have long been recognized to be.

The localization of substances within cells, to which I am to give particular attention, often clearly indicates the function of a particular part of the cell. There are two main ways by which such a qualitative chemical analysis may be carried out: one may deal with isolated masses of a particular structure, as Miescher did with nuclei, or one may by direct microscopic observation visualize the substance within a single intact cell.

Improved methods of isolation have added much information about the chemical composition of nuclei, mitochondria, and microsomes of animal cells and the plastids of plant cells. The sample may be large enough so that a variety of analytical procedures can be carried out to give a comprehensive average composition of structures from a population of billions of cells. If one performs the arduous task of analysis of all fractions in the isolation procedure, as Doctor Hogeboom and his collaborators (2) first did, it is possible to make the very important demonstration that a chemical compound, or a specific activity, is restricted to a particular category of cell component. Thus, it has been repeatedly demonstrated that the desoxyribose nucleic acid of a mass of tissue is all recoverable in the isolated nuclei. Furthermore, if one counts an aliquot from the sample, he can estimate the number of components in the sample, and by division of the figures from the analysis of the sample arrive at an average amount per component. Boivin, Vendrely and Vendrely (7) first did this with isolated nuclei from a variety of beef organs and from spermatozoa. They noted the average amount per tissue nucleus was the same for all organs, and was approximately twice that of the spermatozoon (table 1). Since this was identical with the relationship between the double set of chromosomes of the tissues and the single set of the spermatozoon, they concluded that the amount of nucleic acid was constant for each chromosome set. Davidson and Leslie (10) have suggested that with the DNA determination as a base, one can estimate the number of cells in a sample, and by reference to this express other analytical results as amount per cell. This will, of course, indicate the real situation within each cell only if there is reason to suppose that all cells are alike. The method must therefore often tend to obscure the very aspect of chemical composition which may be most important, namely the variation of cellular composition with cyclical or progressive cell phenomena.

Most workers seem to be well aware of the fact that there are further difficulties in extrapolating to the cell, which arise from the procedures used in isolating components. The purity of the isolation is always problematical, and even though the fraction may obviously contain only a negligible contamination with other structural elements, there is always the possibility that material may be either washed out of the components or adsorbed from other sources.

TABLE 1. DNA PER NUCLEUS IN BEEF TISSUES

ORGAN[1]	AMOUNT[1] ($\times 10^{-9}$ mg.)	NO. OF NUCLEAR TYPES	PROBABLE CHROMOSOME NO.
Thymus	6.4	1	Diploid
Liver	6.4	2	Diploid
Pancreas	6.9	6	Diploid
Kidney	5.9	10	Diploid
Sperm suspension	3.3	1	Haploid

[1] After data of Vendrely and Vendrely, 1948 (8). The major number of nuclear types are estimated from common textbook descriptions of the histology of the organs. Table reproduced from Pollister, Swift, and Alfert, 1951 (9).

Assuming that these difficulties are obviated, the best that the method of isolation alone can yield is data as to the overall average composition and the restriction of a substance to some members of one category of cellular component; it obviously falls far short of giving specific information at the level of a single cell. Thus, from the data of table 1 alone, one could not say whether there might not be in any sample a considerable, though constant, fraction of the nuclei which had no desoxyribose nucleic acid at all. As a matter of fact, such a question has not, so far as I know, ever been raised, because, as everyone knows, there is excellent cytological evidence from application of the highly specific Feulgen reaction for desoxypentose that every single nucleus contains an abundance of desoxyribose nucleic acid. While, as Dr. Hogeboom will tell you, chemical analyses indicate that certain important intracellular respiratory mechanisms appear to be confined to the mitochondrial fraction, it by no means follows that these occur in every individual mitochondrion. The most conclusive evi-

dence that this is so appears to come from the cytological observation that when cells are stained vitally with a dye Janus Green B, which is readily reducible to the colorless leucobase, every mitochondrion remains brilliantly colored in sharp contrast to the colorless remainder of the cell. I respectfully request that you keep in mind for a few minutes these excellent illustrations of the interdependence of the isolation and cytological techniques, while I describe our efforts at adaptation of some chemical methods to the study of individual cells. Compared with the certainty and variety of the analytical techniques which can be used upon a sample of several milligrams of isolated components, our resources will seem extremely limited. Few though they are, however, these are the only gateway which certainly leads directly inside the individual cell.

The possibility of applying analytical methods to achieve the visualization of substances *in situ* within the cell has intrigued many cytologists, histologists and pathologists, and there have been numerous sporadic outbreaks of enthusiasm for one or another supposedly satisfactory method, the devotees of which have proceeded to describe the localization of the particular substance in a wide range of cell types. Many of these are evaluated critically in Lison's *Histochemie Animale* (11). The ideal localization is of course that which can be carried out upon the intact living cell, but in practice this is necessarily limited to substances with strong natural absorption, which occur in relatively high concentration. When this is visible color, like that of chlorophyll or hemoglobin, visualization is obviously easy. It has long been known that with ultraviolet light, absorption within the living cell is very pronounced in the chromatin of the nucleus (fig. 2*D*). In 1936 Caspersson (15), in his masterly doctoral dissertation, proved that the chief basis of this was the natural high specific absorption of the purine and pyrimidine components of the nucleic acid of chromatin. Originally this seemed to offer an opportunity for localization of nucleic acid within living cells, but it is now becoming generally recognized that the interpretation of the ultraviolet absorption spectra of living cells is by no means unequivocal. A great variety of intracellular substances (for example nearly all aromatic compounds) absorb the same part of the ultraviolet spectrum as does nucleic acid, and there seems no way of distinguishing among these in the living cell. Other difficulties arise from non-specific light loss and from the injurious action of the radiation (see Ris and Mirsky, 16). Consequently it is clear, as Caspersson himself has stated in his latest publication (17), that living cells are as a rule unsatisfactory objects for ultraviolet absorption analysis. It is far better to analyze fixed tissues, in which the specificity of the ultraviolet absorption can be checked by subjecting the cells to a variety of solvents and enzymes, as well as to confirmatory specific color tests and stains.

We must then, work mainly with what is called fixed material, and our efforts must be of course confined to those analytical procedures which will not alter the cellular structure beyond recognition. This immediately excludes a great many methods which are too drastic to apply to sections. A further limitation arises from the fact that during the process of fixation there is abundant opportunity for loss of and extensive redistribution of inorganic and smaller organic molecules. This has been repeatedly demonstrated to take place, and the necessity for caution in interpreting the results of microchemical tests for these substances has been frequently stressed.[1] Thus, localization is practically limited to the compounds of large molecular size, which are precipitated or coagulated by fixation. By some special methods certain lipoidal substances can be rendered insoluble, but in most cells the common acid fixation may be assumed to leave essentially unchanged in amount and distribution only the proteins and nucleic acids. These are, in fact, the basis of the common histological picture of the cell, in which the nucleus is stained with basic hematoxylin, the cytoplasm with acid eosin. From the standpoint of nucleoprotein analysis, such fixation is of great advantage, for it is a sort of preliminary fractionation. The specific methods of demonstrating the distribution of protein and nucleic acids in the glandular cell, which was described previously, will illustrate the modern application of qualitative chemical cytology.

Analyses of animal tissue have shown that the bulk of the cellular material, roughly 60 to 80 per cent of the dry weight, is protein. This pro-

[1] Localization of certain mineral elements does seem possible if frozen-dried material is ashed (see Scott, 18) and many other elements can be localized by x-ray absorption analysis of frozen-dried tissue. Also the method of radioautography can probably be extended to the levels of intracellular localization.

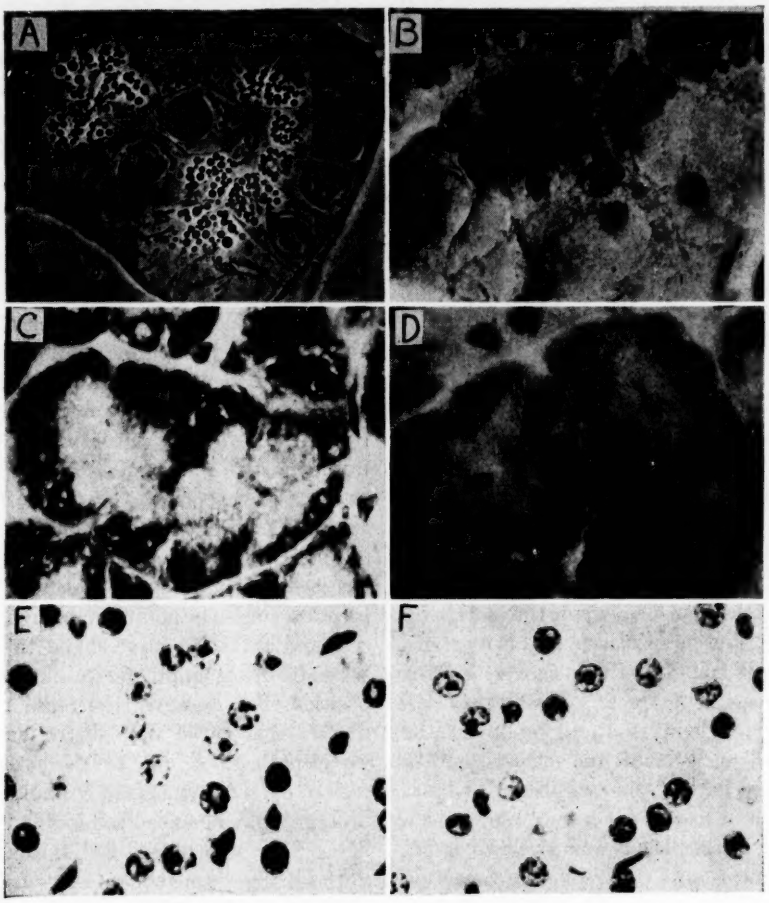

Fig. 2. SERIES of photomicrographs of pancreatic glandular cells, to show structure and the results of various techniques of localization of nucleic acids and proteins.

A. From a tadpole of the green frog, *Rana clamitans*, tissue well fixed to show all structural details. Technique: Double fixation in osmic acid followed by formalin; embedded in n-butyl methacrylate and sectioned at 1 μ, using a Spencer microtome with slow feed attachment and a glass knife; mounted in Cargille's refractive index oil (n_D 1.460), photographed with Zeiss phase contrast equipment, using a Pointolite lamp, Wratten 58 filter, and Kodak M Plates; × 800. *B*. From the salamander, *Necturus*, after the Millon test for protein. Technique; Fixed in Champy's osmic-chromic-bichromate mixture, embedded by the methyl benzoate-celloidin-paraffin method, and sectioned at 6 μ; Millon reaction for total protein as described in Pollister (12); photographed on Wratten M plates, using an AH4 type mercury vapor lamp, from which the 365–6 radiation was isolated by Corning glass filter No. 5840 in 2.4 mm. thickness; × 850. *C*. From an adult male white rat, after staining with the basic dye Azure A with a method which is specific for chemical staining of nucleic acids. Technique: Fixed in acetic alcohol (1:3), embedded in paraffin, and sectioned at 5 μ; stained as described in (12); photographed on Kodak M plates, using a tungsten ribbon filament lamp and a Bausch and Lomb daylight glass filter. Magnification × 800. *D*. From the same block of tissue as *C*, photographed by ultraviolet light to show the high absorption in regions of nucleic acid concentration. Technique: Sectioned at 3 μ and mounted on a fused quartz slide; paraffin removed with chloroform, and section mounted in glycerin saturated with chloral hydrate, as recommended by Kohler (13); photographed on Kodak Process plates, at 254 mμ, with the apparatus described by Pollister and Moses (14), using a Cooke, Troughton, and Sims 81× monochromatic objective; × 800. *E*. Adjacent section to *C*, which has been digested with ribonuclease before staining with Azure A. Technique: Digestion for 2 hrs. at 37°C with Worthington's crystalline ribonuclease, concentration 0.2 mg/ml. p_H 6.0, boiled before use with saturated $(NH_4)_2SO_4$ to destroy protease activity; staining, photography, and magnification as in *C*. *F*. An adjacent section to *C*, after the Feulgen reaction for desoxypentose. Technique: Feulgen reaction as described in (12), photographed as in *C*, using a Wratten 58 filter instead of the daylight glass.

tein is the basis of the staining of cytoplasm with acid dyes, like eosin. A more satisfactory chemical method of showing intracellular distribution of protein is by use of a protein test, like the Millon reaction, which is specific for tyrosine and tryptophane. As figure 2*B* shows, there is protein throughout all parts of the cell, with especially conspicuous concentrations in the zymogen granules and the nucleolus. Such a photomicrograph as this has no chemical meaning unless it can be shown that the method is specific for protein. It is a major distinction between the attitude of many cytologists of the past and of those of today that this point is always critically examined. That the Millon color on slides is formed with the reactive protein groups is demonstrated by the fact that protein masses which are known to be deficient in tyrosine and tryptophane, such as collagen and protamine, are Millon-negative on slides in which adjacent proteins are strongly positive.

The old method which demonstrates the intracellular distribution of nucleic acid is by staining with an acid solution of a basic dye, such as Azure A, shown in figure 2*C*. Histologists call this basophilia. There is strong stain in the chromatin and nucleolus of the nucleus, and in the broad basal part of the cytoplasm. The last reaction has been known for a long time, and a special name, *ergastoplasm*, was given to it before the chemical basis of the staining reaction was understood. Strictly speaking there are two sorts of strongly acid basophilic substances in cells, the phosphoric acid esters which include the two nucleic acids and the sulphuric acid esters which include such substances as mucopolysaccharides. The latter are relatively rare and easily distinguished from nucleic acid when they are present. For precise characterization of the basophilic material in any part of the cell further cytological tests are always necessary. For example, after a companion slide to the last has been digested by ribonuclease the basal zone and nucleolus are no longer stainable with Azure A, while the chromatin still stains as heavily as in an undigested slide (fig. 2*E*). If the ribonuclease digestion is followed by desoxyribonuclease, then the slide will not stain at all with Azure A.[2]

[2] It should be pointed out that it is dye-binding capacity which the Azure A staining indicates. This property depends to a certain extent, perhaps variable from tissue to tissue, upon the association of the nucleic acid with protein; hence a weak or negative basophilia does not necessarily always indicate a low concentration of nucleic acid.

That the nucleic acid of chromatin is largely if not entirely of the desoxypentose type is confirmed by the highly specific Feulgen reaction for this sugar. With this method, as with the azure stain after ribonuclease, there is only a nuclear pattern to indicate the cellular structure of the pancreas (fig. 2*F*). As would be expected, after predigestion of a slide with desoxyribonuclease the Feulgen reaction is negative.

In addition to the basophilia due to phosphoric acid groups of nucleic acid and the Feulgen reaction of the desoxy-sugar the strong natural absorption of the purine and pyrimidine bases has proved to be, in Caspersson's hands, a useful method of localization of polynucleotides. Figure 2*D*, which is a companion slide to that from which 2*C* was photographed, clearly shows, as did the basophilia, the nucleic acid localization in the chromatin, nucleoli and basal zone of ergastoplasm. The method of Caspersson (17) has been to measure complete ultraviolet absorption curves of points in different parts of the cell, and from the curve shape to draw conclusions about the approximate nucleoprotein composition. For example, the basal zone shows a nucleic acid curve with distortion of the long wavelength slope due to the protein; while a region of the zymogenic granules shows only protein ultraviolet absorption. As with basophilia, it has been shown (e.g. by Davidson, 17a) that nuclease digestion reduces specifically the ultraviolet absorption of areas like either the basal zone and nucleolus or of the chromatin, while absorption in all these regions is reduced by pretreatment of the section with both enzymes, or with hot trichloracetic acid. Unlike basophilia, the ultraviolet light loss is by no means all due to specific nucleic acid absorption; a certain amount at any wavelength is due to protein absorption, and often as much as half of the light loss is non-specific because of scattering and internal reflections in the dense protein mass (1, 17).

The variety of techniques which may be used to localize nucleic acids within the cell may be briefly summarized: both pentose and desoxypentose nucleic acid are basophilic, absorb ultraviolet light strongly, and are soluble in hot trichloracetic acid; the specificity of the nucleases and the Feulgen reaction serve to demonstrate whether the nucleic acid in a given region is predominantly of the pentose or the desoxypentose type.

Application of these nucleoprotein methods has completely clarified the questions of distribution

of nucleic acids in cells. Independently Caspersson and his fellow-workers, using ultraviolet absorption and the Feulgen reaction, and Brachet and his associates (19), using basophilia, ribonuclease, and the Feulgen reaction, have made it clear that: *a*) chromatin is the site of major intracellular concentration of desoxyribose nucleic acid; *b*) ribose nucleic acids are especially characteristic of the cytoplasm and the nucleolus; and *c*) that there are striking changes in amount of ribose nucleic acid in cells in which protein synthesis is expecially rapid, as in growth or synthesis of a glandular product. The cell shown above had completed synthesis of a batch of granules; if this were about to begin it would have been noted that the basophilic ergastoplasm would have been much increased, extending distally into the region level with the nucleus.

The qualitative cytological methods which have just been discussed lead to localization of a substance within a cell by its absorption, which is detectable visually. Cytologists usually speak of the intensity of a stain or color reaction as weak, strong, very dark, etc.—and, of course, these terms imply semi-quantitative evaluation of the concentration of the component which is responsible for the color. When two objects are side by side in an evenly illuminated microscopic field, or in the two fields of a comparison eyepiece, visual matching appears to be as accurate as objective photometric measurements. With objects of the same size, proper visual comparison is then a rough indication of relative amounts; if two objects are equally dark they may be assumed to have approximately the same *amount*, and if they appear different the darker one may be taken to contain more reacting substance. The same conclusions regarding relative concentrations may be drawn of two bodies of equal vertical thickness (equal absorbing path). More often, the quantitative question which faces a cytologist cannot be answered even roughly by visual comparison. For example, one often wishes to know relative amounts in two objects of very different size. It is uncertain to what extent by visual study a microscopist can even determine whether two such unequal objects have the same intensity (a rare condition probably); for the relative sizes of the contrasting surroundings introduce considerable difficulty. If this match could be accurately made, then from the dimensions of the object one could compute a fairly good estimate of how much more substance the larger object contained.

The above examples illustrate the range of visual microscopic comparison. If, to cite a very common experience, one cytological object is larger and more lightly colored than another, the cytologist is almost completely helpless to answer the obvious question of whether the decrease of color is entirely due to dilution in the larger mass. The relation of volume to light absorption is easily computed from an actual figure, a measurement of extinction, but such a quantitative datum is absolutely necessary. No amount of experience can train a cytologist's eye to operate as an objective microscopic photometric device. These measurements must be made with objective photometers.

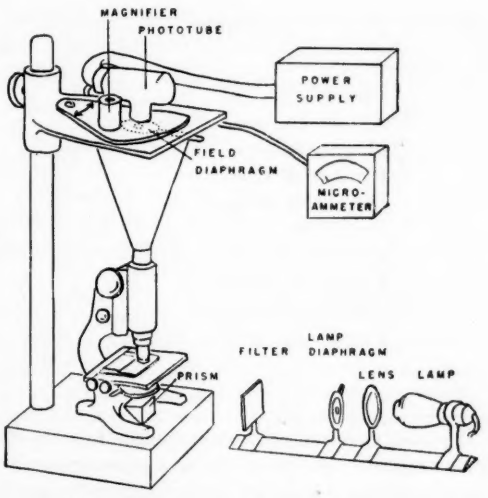

Fig. 3. DIAGRAM of a simplified apparatus for photometric measurements of cytological preparations. After Swift (20).

At the Columbia Laboratory we have for some time been exploring the possibilities of escape from these limitations of visual estimation by adapting quantitative photometric analysis to cytology. We hoped to be able to substitute objective relative values for the differences in absorption which the eye or a photographic plate detects as different intensities. The photometry can be made by carefully controlled photography and densitometry, but it is easier and more accurate to measure the absorption directly by a phototube mounted in the optical axis directly above the microscope (fig. 3). Measurements are made at a single wavelength, near the absorption maximum.

If methods of demonstration of nucleoprotein are to be used in this quantitative fashion ob-

viously they must, in addition to being specific, be reproducible to a known extent. For example, the staining technique must be standardized so that it comes to a sort of end point, always reaching approximately the same intensity with a given sort of cytological material. When I started to measure cell structures it at once became apparent that the individual absorption measurements on any object within a single section were variable over a considerable range—a situation which has likewise been found by all other workers who have given attention to the question. The data for any one object group themselves into that from cell counts (table 1) Boivin and the Vendrelys (7) concluded that the nuclei of tissue cells of any one species of animal all contained approximately the same average amount of DNA. This was fully confirmed by Swift's (20) photometric measurements of individual nuclei of 11 different tissues of the rat (table 3). Essentially similar results have been reported by Pasteels and Lison (23) and by Leuchtenberger, Vendrely and Vendrely (24). It does indeed appear that specializations of cells do not involve major changes in amount of desoxyribose nucleic acid.[3] Swift's photometric measurements also confirmed

TABLE 2. DNA, HUMAN ERYTHROBLAST NUCLEI, FEULGEN

CASE NO.	DIAGNOSIS	BEFORE B_{12}		AFTER B_{12}	
		No. nuc.	E_{550}	No. nuc.	E_{550}
1	Normal	30	.231 ± .003		
2	Nutritional macrocytic anemia	20	.250 ± .009	30	.247 ± .007
3	Megaloblastic anemia of infancy	30	.244 ± .007	5	.244
4	Pernicious anemia	10	.232 ± .007	20	.242 ± .005
5	Pernicious anemia	35	.249 ± .005	27	.238 ± .005
6	Pernicious anemia	30	.240 ± .004	35	.240 ± .005
7	Pernicious anemia	35	.231 ± .006	20	.235 ± .007
8	Pernicious anemia	50	.248 ± .005	20	.242 ± .006
9	Pernicious anemia	40	.256 ± .006	30	.248 ± .005
10	Pernicious anemia, juvenile	30	.231 ± .005	40	.237 ± .006
Mean			.241	Mean	.241

Mean extinction values and standard errors of the mean, Feulgen-stained erythroblast nuclei, smears of human marrow, all nuclei 9.5 μ in diameter. The extinction, E_{550}, is a measure of the amount of nucleic acid in the nuclei. From data of Reissner and Korson, 1951 (22).

fairly normal looking unimodal distribution curves (21). Reproducibility of one of these cytochemical methods must therefore always be estimated by statistical analysis. The shapes of the distribution curves are the same from slide to slide, but reproducibility is more easily assessed by comparing mean values for each population measured. The methods now in use in our laboratory show means which are reproducible within about 10 per cent of the total extinction, as shown for example in Reissner and Korson's data (22) on the Feulgen reaction in human erythroblasts (table 2). Using this simple statistical method of studying slides, a change of composition is detected as a significant difference in mean value.

The results with this quantitative cytology have been most striking when applied to the cell nucleus, especially in measuring its content of desoxypentose nucleic acid. It will be recalled the view of the constant relationship of DNA to number of sets of chromosomes and strengthened the validity of it by showing that polyploid nuclei with 4 and 8 sets of chromosomes had a corresponding greater amount of desoxyribose nucleic acid. Additional cytological researches have traced the synthesis of DNA in animal cells through the cycles of mitosis, meiosis, and fertilization (see Swift, 20; Alfert, 25; Pollister, Swift and Alfert, 19).

These results based upon measurement of the Feulgen reaction not only provide strong con-

[3] But it must also be stressed that the cytological values are means of a population in which the highest computed value may be as much as 50% larger than the lowest. Part of this is undoubtedly due to technical difficulties, such as the inhomogeneities of the absorbing mass, but it is quite possible that there is also a considerable actual variation in DNA content from nucleus to nucleus.

firmatory evidence of one major aspect of the significance of desoxyribose nucleic acid in cellular biology but also validate the whole quantitative photometric approach very satisfactorily. If a biochemist is to use a colorimetric method of analysis, an essential calibration procedure is to set up a series of known concentrations, or standards, and to check how accurately his colorimeter readings reflect the known relative concentrations. With cytological photometry this cannot be done. Unquestionably within the cell the nucleoprotein mass is very different, in physical

TABLE 3. DNA CONTENT OF CLASS I NUCLEI OF RAT TISSUES

CELL TYPE	NO. OF NUCLEI MEASURED	DNA-FEULGEN ARBITRARY UNITS $\left(\frac{Er^2}{F}\right)$
Liver	21	3.34 ± 0.05
Pancreas	20	3.10 ± 0.06
Thymus	33	3.28 ± 0.06
Lymphocytes	19	3.20 ± 0.08
Sertoli cells	18	3.00 ± 0.12
Kidney epithelium	30	3.14 ± 0.04
Epithelium, small intestine	20	2.97 ± 0.04
Spleen	33	3.12 ± 0.04
Motor neurones	20	3.14 ± 0.07
Testicular interstitial cells	20	3.05 ± 0.08

After Swift, 1950 (20). In the formula for computing arbitrary relative values, column 3, E is extinction at 550 mμ, r is the radius of the cylinder measured through the center of the nucleus, and F is the fraction of the total nuclear volume which the central cylinder represents.

state and in concentration, from that when it is studied *in vitro* after extraction from the cell. This difference must have a profound effect upon not only natural absorption but also upon the course of tests and stains for the protein and nucleic acid. For further discussion of this point consult Pollister, (1). For a cytological calibration which will be comparable with that of an analytical biochemist we must look for a natural condition in which there is a wide range of concentrations. From the averages obtained by cell counts and gross analyses (table 1) it is clear that nuclei of non-dividing tissue cells may present just this desired situation. For, if a mixture of several types of nuclei of widely varying volumes (as in the kidney) show nearly the same average DNA value as does a population of nuclei which is nearly homogeneous as to size (as in the thymus) then it must be true that in the larger nuclei the DNA has been diluted in proportion to the increase in cell volume. It is as if a biochemist had prepared his samples, not by dilution of one concentration, but by weighing out 10 one-milligram samples and dissolving them in a range of volumes from 1 to a 100 milliliters. The largest diploid nuclei which Swift analyzed cytologically were nearly 20 times the volume of the smallest. Since the computed DNA was approximately alike in the two nuclear types it is evident that the method can satisfactorily estimate relative DNA over a concentration range from 1 to 20.[4]

TABLE 4. NUCLEASE DIGESTION OF DNA IN NUCLEI

CYTOCHEMICAL METHOD	PART OF NUCLEOTIDE MOLECULE MEASURED	PERCENTAGE REDUCTION OF EXTINCTION
Natural UV absorption	Pyrimidine or purine	39.9
Feulgen reaction	Desoxypentose	39.6
Azure A basophilia	Phosphoric acid	37.7

Measurement of the effect of ribonuclease digestion (2.0 mg/ml.), at high temperature (56°C.), on 5μ sections of the nuclei of liver cells of the salamander, *Amblystoma*, showing the same reduction of extinction, measured by 3 different cytological photometric methods. (Note: such digestion of DNA by ribonuclease is very exceptional; as a rule ribonuclease preparations are highly specific for ribonucleic acid.)

A careful analytical chemist often checks his results with one method by comparison with others. This is readily done with the variety of techniques for estimation of DNA by cytological photometric analysis. Table 4 shows that when there was about 40 per cent reduction of the extinction of the Feulgen reaction, in this experiment with ribonuclease at high temperature, this

[4] The average amount per diploid rat nucleus has been given from cell counts as 5.5×10^{-9} mg. (Leuchtenberger, Vendrely, and Vendrely, 24). From this it may be computed that the average concentration within the normablast nucleus (volume 24 μ^3, after Swift) was of the order of 2.3×10^{-10} mg. μ^3 (23%), and that within the nucleus of the motor neurone (volume, 460 μ^3) the DNA concentration was 1.2×10^{-11} mg. μ^3 (1.2%). By contrast, about the highest concentration of DNA which is readily measurable by ultraviolet absorption in a cuvette one centimeter thick is less than 0.01%.

loss of DNA was also independently determinable by measuring either the capacity of the phosphoric acid to bind Azure A, or the natural ultraviolet absorption.

The results which I have just been discussing were all expressed in arbitrary units, either extinctions or values computed from the extinctions, taking into account the geometry of the object measured. If the relation of the intensity of the reaction measured to the absolute amount of substance were known, from the extinctions the absolute amounts could have been readily computed. Very early in this work Ris and I (26) published what appeared to be this sort of validation of ultraviolet absorption measurements of individual nuclei—but this now appears to have been no more than the result of a fortuitous combination of errors. From the results of Swift and others it is apparent that in rat tissues, the Feulgen reaction bears a constant relationship to the DNA content, but it is not known to what extent this relationship holds from species to species. It is obvious, of course, that of itself the absolute amount in a given cell or cell component is of limited importance; most of the problems can be adequately solved if relative values can be determined.

The work on the desoxypentose nucleic acid content of animal nuclei has combined biochemical and cytological approaches to give a picture of this component of the nucleus as fairly constant, markedly altered only when the chromosome number is changed or the material for new nuclei is synthesized. A similar interdependence of the two approaches is leading toward clarification of the problem of the total nucleoprotein composition of nuclei (27). The older analytical data indicated that even in liver nuclei the protein content was not more than two or three times that of nucleic acid. The available cytological data, by contrast, showed indications that the protein content was much higher, up to 5 to 20 times the nucleic acid. Leuchtenberger and I assumed that this meant that the washing incidental to the isolation of nuclei removed a considerable amount of the protein; and we showed by photometric measurements of nuclei in fresh slices of liver that such loss did indeed occur. From other analytical data, most of them more recent, and using different isolation procedures there may be computed considerably higher protein values which are in some cases (28) even higher than those we gave for liver. We now know that our ratios were probably high by a factor of two, but even with this correction the protein content is much higher than that from the older analyses. From the gross analytical data alone it could not be decided whether the low values were a result of loss of protein, or whether the high values indicated that protein had been adsorbed onto the nuclei from the surrounding cytoplasm. The comparison of the cytological and biochemical data, however, makes it highly likely that the nucleus does, as a rule, contain high protein concentration, quite comparable with that of the cytoplasm.

In closing, I should like to summarize briefly. The attempt to localize substances in cells is one approach to the broad problem of determining to what extent cellular structure reflects diversity in distribution of the physiological functions of the cell. Localization of substances can best be achieved by a combination of two approaches; analysis of isolated masses of cell components and direct localization by microscopic study of cells. In most cases the cytological study must be made on fixed cells, on which a variety of specific tests, stains, digestions, and chemical extractions can be carried out. Cytological localization methods are best developed for proteins and nucleic acids, and these have been adapted to yield quantitative estimations of relative amounts in cells. The quantitative techniques have been used most successfully in study of the composition of the cell nucleus.

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SEPARATION AND PROPERTIES OF CELL COMPONENTS[1]

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CERTAINLY the most versatile approach to an understanding of the relation between cell structure and cell chemistry is the procedure of cell fractionation, whereby the cell is mechanically broken and its structural elements are segregated according to size and density by fractionation in the centrifuge. The main advantage of this procedure lies in the fact that cell fractions can be obtained in sufficient amounts to permit the direct application of quantitative biochemical methods of analysis as fast as these methods are developed. To the best of my knowledge, no other general cytochemical technique has this capability.

It is, therefore, simple enough to prepare a broken cell suspension, subject the suspension to increasing centrifugal force, isolate particles of various sizes, and carry out chemical analyses on the fractions obtained. Past experience has amply demonstrated, however, that it is not simple to arrive at firm conclusions relating the chemical data to the structural make-up of the cell, and this is the point where the cell fractionation procedure must be examined critically. Now what is liable to happen when the cell membrane is broken? First, the intracellular structures are dispersed into an aqueous medium bearing only a faint resemblance to that within the living cell. When so dispersed, the structural elements behave appropriately by displaying a pronounced tendency to undergo morphological alteration (1). Aside from the fact that this phenomenon of morphological alteration can make it difficult if not impossible to identify cytologically the dispersed structures and thus to determine the composition of isolated cell fractions, a number of other undesirable phenomena might be expected to follow, such as a redistribution of biochemical properties as a result of leakage of substances through damaged membranes, or adsorption of soluble compounds on particulate material.

These considerations have led to the establishment of several criteria defining an adequate isolation procedure, the first and most obvious of which is that a sound cytological background must be provided. Thus the morphological integrity of the cell structures must be preserved to such an extent that the composition of the isolated fractions can be directly determined by cytological study. It seems reasonable to believe that this principle also has some bearing on the possible occurrence of redistribution of biochemical properties after cell disruption. Thus the membrane of a cytologically intact structure, for example nucleus or mitochondrion, would probably be less likely to leak than the membrane of an obviously altered structure.

A second item of importance involves the question of yield. To isolate only a small proportion of the nuclei or mitochondria of a given tissue and attempt to interpret results obtained with that preparation in terms of the whole tissue is an extrapolation of questionable validity, particularly when one considers the possibility of heterogeneity of cells and perhaps even of nuclei or of mitochondria. It is therefore necessary to provide a means by which almost all of the cells of a tissue can be broken and to devise a fractionation procedure permitting reasonably clean separation of the various cell components and their isolation in good yield.

Two important additional points arise in connection with the biochemical analysis of the fractions and the interpretation of the biochemical data. First, it is essential to analyze both the original whole tissue and all the fractions obtained from it. By this means it is possible to draw up a balance sheet, which serves as a test of the validity of the analytical method, enabling one to detect the presence of inhibitors or activators or the possible role of more than one cell

[1] For the sake of brevity, the following abbreviations will be employed: DPN = diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide; ATP = adenosine triphosphate; ATPase = adenosine triphosphatase; AMP = adenosine-5-phosphate; AMPase = AMP phosphatase; DNA = desoxypentosenucleic acid; PNA = pentosenucleic acid.

[2] Federal Security Agency, U. S. Public Health Service.

component in a given biochemical reaction. The second requirement arises from a consideration of the possibility of adsorption of soluble compounds on particulate material and of an overlapping of fractions that may not be readily detected by cytological study. Thus in an investigation of the distribution of an enzyme among cell fractions, the presence in a single fraction of a relatively small proportion of the total enzyme should be interpreted with caution. The finding that the enzyme is concentrated in the fraction to a greater extent than in the original whole tissue is much more likely to be significant from the cytochemical standpoint.

Up to the present time, mammalian liver has been by far the most popular tissue for cell fractionation studies. Liver presents certain definite advantages over most other tissues. It is largely composed of what appears to be a single cell type and the individual liver cells are large and easily disrupted. The abundant cytoplasm contains many mitochondria (probably somewhere in the neighborhood of 700[3] per cell), and apparently several types of submicroscopic structures which have recently been revealed by electron microscopy. Also present in the cytoplasm and visible in the light microscope are lipid droplets and a number of small spherical secretory granules which stain with neutral red and are usually located near the periphery of the cell. The cell contains a single nucleus containing 1 to 3 nucleoli.

By utilizing the mitochondrion as a test object, it has been possible to obtain a considerable amount of information concerning the proper conditions for fractionating the liver cell. The mitochondrion, which has almost as widespread a distribution among cells, plant and animal, as the nucleus, has certain characteristic cytological properties, including a usually elongated shape, the probable presence of a surface membrane, specific staining vitally with Janus Green B and after special types of fixation with certain other dyes, and a very pronounced sensitivity to mishandling. In the latter respect, both the cytological and biochemical properties of mitochondria are markedly affected by the composition of the suspending medium. The following facts became apparent in a study of the effect of various media on mitochondria (1). The use of water or other hypotonic solutions causes prohibitive morphological alteration, and the use of solutions of electrolytes at the isotonic level produces aggregation of the particles to such an extent that they cannot be adequately separated from nuclei. An answer to these problems of morphological alteration and of aggregation was found, however, in the use of solutions of nonelectrolytes. Two media evolved from the study: isotonic (0.25M) sucrose in which the mitochondria change from an elongated into a spherical shape but otherwise retain their normal cytological properties, and hypertonic (0.88M) sucrose in which the particles retain both their normal shape and staining properties. Subsequently, a comparative study of these two media has led to the conclusion that neither possesses any distinct advantage as far as the distribution of biochemical properties among the cell fractions is concerned. There are, however, difficulties associated with the use of hypertonic sucrose, including the necessary addition of large amounts of sucrose to reaction mixtures and, because of the high density and viscosity of the solution, a requirement for high centrifugal forces in the fractionation procedure. For these reasons we are at present using the more convenient of the two media, namely the isotonic sucrose. Perhaps I should point out, however, that we do not by any means feel that the choice of a proper medium for cell fractionation is a closed problem. Sucrose solutions are certainly far from physiological in the sense of duplicating the medium of the cytoplasm, and we are well aware of the possibility that artifacts may eventually be encountered as a result of the use of such unphysiologic solutions. The addition of various salts at low concentrations to the sucrose solutions in an attempt to improve the physiological characteristics of the media has, in our experience, invariably resulted in some degree of aggregation of particles and thus in difficulties in obtaining adequate fractionation.

The fractionation procedure (2) is, of course, carried out entirely in the cold, the temperature of the preparations being maintained at 5° or less. In our experience, the Potter-Elvehjem homogenizer (3) provides the most satisfactory method for disrupting cells, since its use results in the breakage of 80 per cent or more of the liver cells without damage to an appreciable proportion of the nuclei. The Waring blendor is entirely unsatisfactory for the preparation of

[3] This value was obtained by making simultaneous counts of nuclei and mitochondria in homogenates of mouse and rat liver.

homogenates suitable for cell fractionation studies. The subjection of liver, for example, to the action of the Waring blendor for periods sufficient to disrupt most of the cells results in the breakage of many nuclei and even of some mitochondria.

By the use of increasing centrifugal forces, four fractions are obtained from homogenates of mammalian liver; first, a nuclear fraction containing about 15 per cent of the original total nitrogen and including all of the nuclei of the homogenate together with some residual intact cells and a variable but usually small number of free mitochondria; second, a mitochondrial fraction in which virtually all of the visible particles satisfy the cytological criteria for the identification of mitochondria and in which, as indicated by electron microscopy, there are apparently few submicroscopic particles (1). The mitochondrial fraction contains approximately 25 per cent of the original total nitrogen. The third fraction, the submicroscopic particles or microsomes, contains 20 to 25 per cent of the original nitrogen. The types of structures present in this fraction have not as yet been clearly defined, although recent studies carried out with A. J. Dalton with the electron microscope have indicated the presence of several types of particles of diameters ranging between 30 and approximately 150 millimicrons. The fourth fraction, a final supernatant, contains 35 to 40 per cent of the original nitrogen and is made up of the soluble material of the cell; possibly some very small particles, lipid droplets, and some of the secretory granules. It is our experience that most of the secretory granules do not exist as formed elements after disruption of the liver cell (1).

It is apparent, therefore, that this fractionation procedure is suitable for the isolation of the structural elements of the cytoplasm but does not yield a homogeneous preparation of nuclei. In fact, it seems unlikely that a satisfactory method for the isolation of cytologically intact nuclei in good yield has yet been developed. Although the use of the citric acid method, for example, permits reasonably clean separation of nuclei from cytoplasmic constituents, the nuclei so obtained are obviously morphologically altered (4). A similar picture is obtained by the use of the Behrens' procedure (5, 6). Recently, several modifications of the sucrose (7, 8) technique have yielded clean preparations of nuclei that appear cytologically intact, but all these methods thus far permit the isolation of only a small proportion of the total number of nuclei of the tissue.

By far the most striking result of studies of the biochemical properties of cell fractions has been the partial elucidation, by work carried out in a number of laboratories, of the important role played by mitochondria in the metabolism of the liver cell. In this respect, one of the earliest discovered enzymatic properties of mitochondria was their content of cytochrome oxidase and succinic dehydrogenase (9, 10). Several experiments have suggested that these two enzyme systems are, in fact, exclusively a function of mitochondria, the relatively small amount of activity shown by other fractions probably being the result of contamination by mitochondria (1). Other enzyme systems and related substances that are concentrated in this fraction of liver are oxalacetic acid oxidase (11), octanoic acid oxidase (12, 13) glutamic dehydrogenase,[4] DPN cytochrome reductase, TPN cytochrome reductase (14–16), adenosinetriphosphatase (9, 17) catalase (18), uricase (19), cytochrome *c* (2, 20), riboflavin (21), and vitamin B_6 (22). The system catalyzing the synthesis of *p*-aminohippuric acid from *p*-aminobenzoic acid and glycine was largely recovered in the mitochondrial fraction (23). Recently, Kielley and Kielley (24) in an interesting study of oxidative phosphorylation have shown that in the presence of an oxidizable substrate, such as α-ketoglutarate, liver mitochondria are capable of synthesizing ATP from AMP and inorganic phosphate at a very rapid rate, the net uptake of inorganic phosphorus at 28° being approximately 2 μM/min/mg. of mitochondrial nitrogen. In this study it was also shown that the 'myokinase' activity of liver is largely, if not exclusively, associated with mitochondria and plays a prominent role in the phosphorylation mechanism (24). The adenosinetriphosphatase (ATPase) of mitochondria was found to be non-operative under the conditions of ATP synthesis and became operative only when the particles were aged or subjected to procedures causing structural alteration and at the same time a loss of their ability to carry out the phosphorylation reaction. The latter finding brings up the interesting possibility that ATPase may under appropriate conditions actually function as a synthesizing system.

[4] Hogeboom, G. H. and W. C. Schneider. Unpublished experiments.

Of the enzyme systems that are concentrated in mitochondria, several are apparently not exclusively associated with this cellular element. The cytochrome reductases, for example, are also concentrated in microsomes. ATPase is also concentrated in the nuclear fraction, catalase and cytochrome *c* in the final supernatant, and the activity of mitochondria in the oxidation of oxalacetate, and to a lesser extent of octanoate, is enhanced by the addition of other fractions which by themselves are inactive. The situation with respect to α-ketoglutarate oxidation is not entirely clear, since a satisfactory method for the assay of this system in homogenates is not available. Isolated mitochondria, however, are capable of oxidizing α-ketoglutarate in the presence of AMP and magnesium ions at a reasonably rapid rate, the Q_{O_2} in terms of milligrams of total nitrogen at 28° being approximately 400 μl. of O_2/hour (24). It thus seems possible that mitochondria are largely responsible for this reaction.

Another interesting study having a bearing, from the negative standpoint, on the properties of mitochondria, was carried out by LePage and Schneider (25). These investigators found that over 50 per cent of the anaerobic glycolytic activity of rabbit liver was recovered in the final supernatant. No other fraction showed appreciable activity except the nuclei, which, of course, were contaminated by intact cells. When the fractions were recombined, the activity of the supernatant was enhanced greatly by the addition of microsomes but only slightly by the addition of mitochondria. It would thus appear that mitochondria are not intimately concerned with the anaerobic phases of glycolysis.

Another finding of interest from the negative point of view is that mitochondria contain only a relatively small amount of pentose nucleic acid (1, 9, 17). Approximately 25 per cent of the total mass of mitochondria appears to be composed of lipide, of which about two-thirds is phospholipide (26–28).

It seems apparent, therefore, that mitochondria are chiefly concerned with the oxidative metabolism of the cell, including some of the reactions of the Krebs cycle. Perhaps their most interesting biochemical property, arising from the studies on phosphorylation, among others, is their ability to carry out reactions capable of supplying energy for synthesis.

As far as the enzymatic properties of the nucleus are concerned, the results of investigations of the nuclear fraction have been largely negative. Although in all cases some enzyme activity is always shown by the nuclear fraction, only in the case of ATPase (9, 17) and AMPase (29) is the concentration of enzyme sufficiently high to rule out contaminating cytoplasmic elements as the cell structures responsible for the activity. In general, the results of enzyme determinations carried out on nuclei isolated by other techniques are open to the same difficulty in interpretation (4). It is, on the other hand, entirely possible that the nucleus actually does contain a large number of enzymes in relatively low concentration, but proof that this is the case is certainly not a simple problem.

The submicroscopic particles or microsomes are characterized by strikingly high concentrations of pentose nucleic acid (PNA) and of lipide, which is mostly in the form of phospholipide. The concentration of PNA in microsomes is some 5 to 6 times that found in the mitochondrial fraction (1, 11, 12, 17, 28), and approximately 40 per cent of the dry weight of microsomes is lipide in nature (26–28, 30).

Until recently, the metabolic function of microsomes has been largely a matter of conjecture, but during the last few years several enzymes have been shown to occur in the fraction in a concentration exceeding that in whole tissue. These enzymes include both DPN and TPN cytochrome reductase (14–16), the former being present in considerably higher concentration than the latter. Omachi, Barnum, and Glick (31) have reported that the esterase activity of whole liver is largely recovered in the microsome fraction. Microsomes also play a role in certain other enzyme reactions, including anaerobic glycolysis (25), the oxidation of oxalacetate (11), and the reductive cleavage of *p*-dimethylaminoazobenzene (32).

It is obvious, however, that our present knowledge of the role of microsomes in cellular metabolism is quite limited and does not permit the type of generalizations that can be made in the case of mitochondria.

The fourth fraction obtained from mammalian liver, the supernatant, is, of course, a highly complex mixture containing all of the enzymes involved in anaerobic glycolysis (25), as well as isocitric dehydrogenase (16), acid and alkaline phosphatase (29), catalase (18), cytochrome *c* (20), and undoubtedly many other substances of biochemical importance. A detailed study of the types of proteins present in the soluble frac-

tion of the cell is a problem of major proportions in itself. Recently, some steps in this direction have been made by Sorof and Cohen through the use of electrophoretic and ultracentrifugal analysis (33, 34).

Perhaps it would be of some interest if I were to devote the last few minutes to a discussion of recent experiments designed to investigate further the properties of liver mitochondria. For a time it was somewhat disturbing to find that practically all of the enzyme systems associated with mitochondria were 'insoluble', i.e. easily sedimented from extracts of practically any tissue, not obtainable in a monodisperse state in true solution, and thus refractive to extensive purification. Notable examples were cytochrome oxidase, succinic dehydrogenase, ATPase, and DPN-cytochrome reductase. In view of evidence favoring the existence of a mitochondrial membrane (35, 36) and in spite of the cytological evidence for the integrity of isolated mitochondria, it seemed conceivable that the membranes of the particles were actually being damaged sufficiently during the fractionation procedure to allow the escape of soluble substances, leaving only an unsoluble residue. Several lines of evidence have clearly indicated, however, that this is not the case. More recent studies have shown, for example, that several soluble proteins that can readily be purified, such as cytochrome *c*, TPN-cytochrome reductase, myokinase, and glutamic dehydrogenase, are concentrated to a considerable extent in the mitochondrial fraction. Finally, when the mitochondrial membranes are mechanically broken (37, 38) either by the use of intense sonic vibrations or by forcing the particles through a small orifice under high pressure, approximately 60 per cent of the total nitrogen is released into solution. Further studies of the soluble fraction of mitochondria have shown that it consists largely of proteins that can be separated and characterized in the analytical centrifuge. Different refractive index patterns are obtained by the two methods of disintegration of mitochondria, the only component that appears to sediment as a monodisperse system being released by sonic vibrations (38). This component, which constitutes a large proportion of the total protein of mitochondria, and is characterized by a sedimentation constant of 6.2 Svedberg units, is apparently either denatured or dissociated when the mitochondrial disintegration is carried out by forcing the particles through a small orifice.

Of some interest is the fact that the soluble fraction of mitochondria isolated from a liver tumor shows a qualitatively different protein pattern in the analytical centrifuge, in that the main component obtained from normal liver mitochondria appears to be absent (38), whereas other components are seen that correspond in sedimentation constant to those of the normal liver mitochondria. Furthermore, the two methods of disintegration produce identical patterns in the case of the tumor mitochondria but strikingly different patterns in the case of normal liver mitochondria. Attempts are now under way to isolate and characterize the component of normal liver mitochondria that cannot be detected in the tumor preparation.

The effect of the disintegration of mitochondria on the enzyme systems of the fraction is also of some interest. Myokinase and glutamic dehydrogenase are released into solution. Cytochrome oxidase, DPN-cytochrome reductase, ATPase, and, somewhat surprisingly, cytochrome *c* remain attached to particles that are sedimented with considerable difficulty,[4] not being completely sedimented by 30 to 40 minutes centrifugation in an angle centrifuge at 150,000 *g*. Moreover, the specific cytochrome oxidase and DPN cytochrome reductase activities of the slowly sedimenting particles are much higher than the corresponding activities of the original preparation of mitochondria. It is obvious that without the availability of a high speed centrifuge permitting sedimentation with a minimum of convection, it could be erroneously assumed that these 'insoluble' enzymes had been brought into solution.

As I have attempted to illustrate, the procedure of cell fractionation, despite certain deficiencies, is capable of yielding a considerable amount of information both in the field of cytochemistry and in basic studies of the mechanism of biochemical reactions. It is our hope that future efforts in this field will emphasize the development of new methods capable of checking independently and extending the present data.

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INTRACELLULAR ENZYME DISTRIBUTION; INTERPRETATIONS AND SIGNIFICANCE[1]

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IT IS now 9 years since the symposium entitled "Frontiers in Cytochemistry" was held in honor of Prof. R. R. Bensley at the University of Chicago and what little we know about enzyme distribution in cells has developed largely since that time, inasmuch as almost no data on intracellular distribution of enzymes were presented at that meeting. Indeed, the first observations on succinoxidase distribution as reported there differ considerably from present views. Nevertheless a beginning was made and the time was ripe for further developments.

A recent review by Schneider and Hogeboom (1) has surveyed the literature on enzyme distribution up to this time, and therefore in covering the subject that was assigned to me for the present occasion, I shall feel free to spend more time on what we take to be the significance of studies in this field rather than to cover such details of enzyme localization as are available. Since this field is just beginning to establish itself, this may be a good time to take stock of our present position and to ask ourselves what we may hope to accomplish by these studies.

There are two approaches that are beginning to be widely applied in the field of intracellular enzyme localization. One is the so-called histochemical method, based on the classical methods of histology, and the other is the method that is based on the early experiments by Bensley and by Claude, namely, the method of differential centrifugation of homogenized tissue samples. We shall not attempt to interpret the findings obtained by the histochemical method except in one instance, in which the application by Novikoff (2, 3) of the method of differential centrifugation to the localization of alkaline phosphatase led directly to the rejection of the widely held view that the enzyme was localized in the nucleus, as indicated by the usual histochemical method. The same view has now been accepted by Gomori (4), and is apparently not a subject of controversy.

The early philosophy of the differential centrifugation school was summed up very neatly by Professor Bensley in the statements that "It would seem to be an axiom of analytic chemistry to separate separable things before proceeding to their analysis," and "The analysis of particulate components of cytoplasm is only as good as the species-purity of the preparation." (5). Although it is apparent that Bensley regarded the immediate task as analytical, he was also well aware of the broader implications of the work. He said, "Obviously the possibility of separating mitochondria and particulates, and of isolating the structural proteins for chemical study, opens a rich field for further research. The localization of enzyme and carrier systems, vitamins and hormones, and the viruses, functional changes in composition, the tracing of radioactive isotopes into the interior of the cell, and the further fractionation of the submicroscopic particles by more refined methods all offer inviting opportunities to the inquiring mind." (5). It is the purpose of the present discussion to inquire whether the hopes and predictions of Bensley were justified, in the light of the subsequent 9 years of experimentation in this field. What are some of the objectives that we may hope to attain by further work in this field?

OBJECTIVES

When looked at as an analytical problem, that is, the determination of enzyme distribution in the obtainable cell fractions, it must be realized that anything less than the quantitative approach has very little significance. Thus the problem of enzyme distribution in cell fractions involves the same hazards and the same precautions that are

[1] The experimental work from the authors' laboratory was supported in part by grants from the National Cancer Institute of the National Institutes of Health, Public Health Service, and from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

met in applying the homogenate techniques to the determination of enzymes in tissues as pointed out in 1944 (6). This means that the reaction mixture must be fortified with such cofactors and adjuvants as may be required to make the given enzyme the rate determining factor. But it must be clear that the enzyme distribution studies are not an end in themselves. It is clear by this time that the localization of enzymes in cell fractions is no longer the main line of development in this field but that the methods of differential centrifugation can now be used as a powerful tool for the study of *intracellular physiology*, which is the underlying basis of physiology at all higher levels.

What can we hope to gain by knowing more about where the individual enzymes in a cell are located? Stated in the broadest terms, these studies have as their objective the understanding of events occurring at higher levels of organization.

In order to understand these events it is necessary to effect a fusion between the morphological or structural aspects of biology and the chemical or metabolical aspects, and to correlate both along the time scale. The understanding of the morphological and metabolic changes at various levels of organization is in our opinion *primarily a matter of understanding the control or regulatory mechanisms that are called upon*. In this connection, we think of enzyme action in terms of two main categories: *1*) the *controlled enzyme activity* that is manifest *in situ* at the highest level of enzyme organization, namely, the intact animal, in which the manifold factors of the internal and external environment are brought to bear, and *2*) the *potential enzyme activity* that the various enzyme systems can be shown to possess at lower levels of organization, such as the homogenate or the cell fraction. In this discussion we hope to show how the methods of differential centrifugation of homogenized tissues can contribute effectively to studies at both levels, and to mention various subsidiary problems that arise.

CONTROLLED ENZYME ACTIVITY IN SITU

An example of how these methods are proving useful for the study of controlled enzyme activity *in situ* lies in the field of nucleic acid metabolism. For some time evidence has been accumulating in the laboratory of Caspersson (7) and elsewhere that important interactions between the nucleus and cytoplasm are involved in nucleic acid synthesis. The studies based on microscopic examination of individual cells have certain limitations for the study of metabolic processes, and various investigators, including Jeener and Szafarz (8), Barnum and Huseby (9) and Marshak (10) have attempted to supplement the findings on single cells with metabolic studies involving isotopic phosphorus combined with techniques of differential centrifugation. Their studies were complicated by the fact that phosphorus can follow many metabolic pathways, but nevertheless valid conclusions were reached as to the high metabolic activity of the nuclear PNA (pentosenucleic acid). I will illustrate this type of experiment with data that are being obtained in

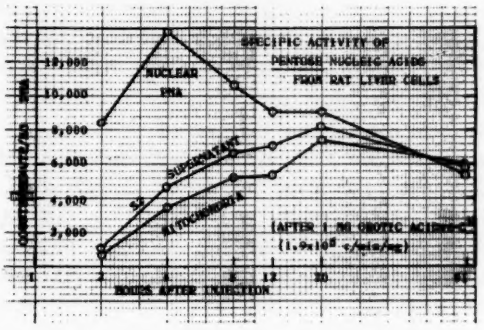

Fig. 1. SPECIFIC ACTIVITY of PNA in rat liver cell fractions.

our laboratory by Mr. Hurlbert. We injected rats with 1 mg. of C^{14} labeled orotic acid (11), which has been shown to be converted into the pyrimidines of nucleic acids in high yield (12–15), while the purines do not incorporate this substance. Rats were killed at time intervals of 2, 4, 8, 12, 20 and 91 hours and the amounts of radioactivity in the internal organs, respiratory CO_2 and excretory products were determined. The livers contained most of the C^{14} retained in the body. They were homogenized and separated into nuclear, mitochondrial, and supernatant (S_2) fractions by differential centrifugation using the sucrose medium as employed by Price, Miller and Miller (16). The PNA and DNA of the nuclear fraction were separated. In the initial period, most of the C^{14} was in the nuclear fraction and was in the pentosenucleic acid (PNA) of this fraction. No C^{14} was detected in the desoxypentose nucleic acid (DNA) at this time. The results (fig. 1) are shown in terms of the specific activities of the PNA of the nuclear, mitochondrial and supernatant (S_2) fractions. From this first approximation of the intracellular distribu-

tion it is clear that the label was first incorporated into the PNA of the nuclear fraction, then into the PNA of the cytoplasmic fractions. After 91 hours, the DNA had attained only a small fraction of the radioactivity in the liver. Thus the suggestions of earlier workers regarding the importance of the nuclear PNA (7–10), considered to reside largely in the nucleolus, is reinforced by these studies, more particularly because instead of using P^{32} the present study was carried out with orotic acid. This substance is incorporated with a comparatively high yield, and has alternative metabolic pathways that appear to be more restricted than those of phosphorus. From these data it is clear that the orotic acid is not being taken up at the same rate by all parts of the cell—it is being taken up by the nucleus, and indeed by a specific portion of the nucleus other than the DNA portion. By following the rates of incorporation of the label from orotic acid into the various cytoplasmic particles along the time scale, and under various conditions which augment the incorporation into the DNA of the chromosomes, it should be possible to test some of the current hypotheses regarding the origin and interrelationships among the various formed bodies found within the cell (cf. 8).

We feel that these data illustrate how the method of differential centrifugation combined with appropriate isotopic precursors can be a powerful tool for the study of the controlled activity of localized enzymes *in situ*, operating at the highest level of enzyme organization. This was the first main category of enzyme activity which we mentioned as an important field for the application of these methods to the problem of the control mechanisms.

POTENTIAL ENZYME ACTIVITY

The second main category in which the methods of differential centrifugation seem at present to be indispensable is the study of potential (and experimentally altered) enzyme activity at the lower levels of organization in terms of intracellular control mechanisms. In studying the activity of the cell components we can observe relationships that enable us to begin interpreting the data obtained by means of slices, whole homogenates and the washed residues, etc. It is now clear that many of the enzymes present in the whole organism or even in the tissue slice do not operate at capacity under usual conditions of study. When these enzymes are studied by the homogenate technique or in isolated cell fractions they will be either *less active* or *more active* than they were *in situ*—less active if the necessary reaction components are in lower concentration than *in situ*, and more active if the reaction components are in optimum concentration. When the enzymes are studied at the level of the homogenate or the cell fractions, it becomes possible to study their requirements for maximum performance while maintaining them in their intracellular form. By learning just what factors are necessary for maximum performance, we are in a position to learn not only the *amount* of the enzyme present in a given cell fraction, but we also learn what factors the other parts of the cell would have to contribute in order to control the activity of the fraction under study. Although there are many ways in which the activity of an enzyme can be regulated (6,17,18), we believe that one of the more important is by the regulation of the supply of substrates and co-substrates (coenzymes), and this is obviously a mechanism in which the spatial distribution of enzymes could be decisive. We refer particularly to the delivery of co-substrates such as inorganic phosphate and phosphate acceptors, which appear to be necessary components and hence common denominators in a large number of reactions, with the result that any reaction that tends to use up the supply of either of these reaction components can shut off or regulate the rate of all the other reactions that are dependent upon the phosphate balance (6, 19, 20). The delivery of co-substrates can also be decisive in determining the choice between alternative metabolic pathways, as we have recently shown in the case of pyruvate metabolism (21).

Let us examine the data obtainable by various techniques. The first point that needs to be emphasized is that the comparison of slices with homogenates under conditions that are appropriate only for slices is a meaningless gesture. When a reaction can be studied in a homogenate, and the optimum conditions are worked out, the reaction tends to be faster in the homogenate than in the slice. In figure 2 the activity of the homogenate is given as 100 per cent, and the activity of the slice is given as a percentage of the homogenate data. Thus the slices compare with the homogenates as follows: urea formation in rat liver, 43 per cent (22); *p*-amino-hippuric acid synthesis in rat liver, 32 per cent (23); liver oxidation of pyruvate, 38 per cent (24. 25); kidney

oxidation of pyruvate, 40 per cent (24, 25); liver synthesis of acetoacetate, 79 per cent (21); and in the case of the only tumor so far studied in these terms, pyruvate oxidation was about equal by both techniques. The possible significance of the latter observation has been discussed elsewhere (26).

It is evident that in many instances the enzymes of the slice are not working at capacity, in comparison with homogenates from the same tissues. Once it is clear that this can be true, it is not too surprising to find reports in which the suppression of an enzyme *in vivo* is so pronounced as to make it appear that an organism lacks the enzyme, although extracts of the organism contain the enzyme in demonstrable form, as in the case of the pantothenicless mutants 5531 and 34556 of Neurospora (27). The more usual case will of course involve only modifications of enzyme activity.

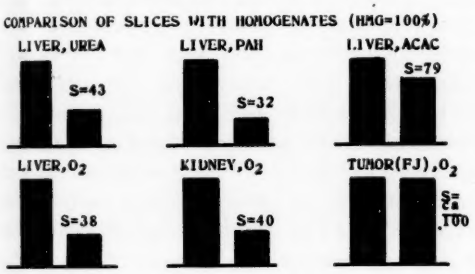

Fig. 2. COMPARISON of slice activity with homogenate activity. Data from references 21–24.

The case of pyruvate oxidation in kidney may be discussed in somewhat greater detail, since studies with cell fractions are available for comparison (fig. 3). The oxidation of pyruvate by rat kidney slices yields a Q_{O_2} of about 17, while that of the homogenate under optimum conditions is about 3-fold greater, or 52 (24, 25). When we examined the components of the kidney homogenates (28) we found that the nuclei and the S_2 supernatant were almost inactive, while the mitochondria possessed only 33 per cent of the activity of the homogenate. When the mitochondria were supplemented by either the nuclei or the supernatant fraction the activity was doubled.

In interpreting the stimulating effects of the other fractions in the presence of the mitochondria, it is necessary to consider what individual enzymes these fractions contain. Hogeboom and Schneider have studied the distribution of cytochrome *c* reductases (29, 30) and isocitric dehydrogenase (30) in the various fractions and find large amounts of the reductases in the microsomes while a high proportion of the dehydrogenase is in the soluble fraction. They were of the opinion that the rate of oxygen uptake by the mitochondria was mainly a measure of cytochrome reductase activity. However, on the basis of the following studies, we believe that in the mitochondria alone the rate-limiting reaction is phosphate breakdown.

We carried out phosphate balance studies on kidney mitochondria, whole homogenates and mitochondria plus nuclei (31). The mitochondria did not break down the added ATP until the substrate was depleted, while the whole homogenate dephosphorylated the ATP much sooner. When nuclei were added to mitochondria, the oxidative rate was doubled and the phosphate breakdown was also accelerated. Since the nuclei do not appear to contain the oxidative enzymes (29, 30) to account for the doubling of the rate, it is suggested that the oxidative enzymes in the mitochondria are adequate but that their potentiality can only be demonstrated by eliminating the rate limiting factor, namely the availability of the phosphate acceptor.

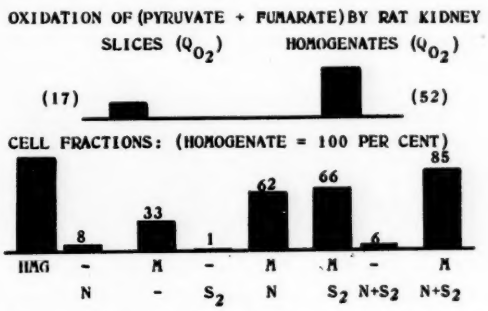

Fig. 3. COMPARISON of pyruvate oxidation by kidney slices, homogenates and cell fractions.

The next experimental step is the substitution of a phosphate acceptor system for the nuclear fraction, as a supplement to the isolated mitochondria, and such studies have recently been reported by Lardy (32) (fig. 4). Mitochondrial fractions obtained from rat liver were supplemented with purified hexokinase or with creatine phosphorylase and the respective phosphate acceptor systems. It was demonstrated that as much as 400 per cent stimulation of oxygen uptake could be obtained, under conditions that are comparable to what we employed in the studies

with mitochondria and nuclei (28, 31). Kielly and Kielly (33) and the Lipmann group (34) have also observed stimulation of oxygen uptake when mitochondria were supplemented with hexokinase and glucose. These studies leave little doubt that the mitochondria are not primarily limited with respect to oxidative enzymes and that their oxidative rate is in fact limited by the rate at which phosphate acceptors are made available. On the basis of these facts we suggest that similar considerations may apply to slices of certain normal tissues, in other words, that the oxidative rate of these normal tissue slices may also be limited by the rate at which phosphate acceptors are made available (cf. 35, 26). This view implies that under appropriate conditions the oxidative rate of these normal tissue slices could be accelerated, and such increased rates of oxidation in tissue slices have of course been repeatedly observed in the presence of dinitrophenol and various other agents (cf. 36, 37).

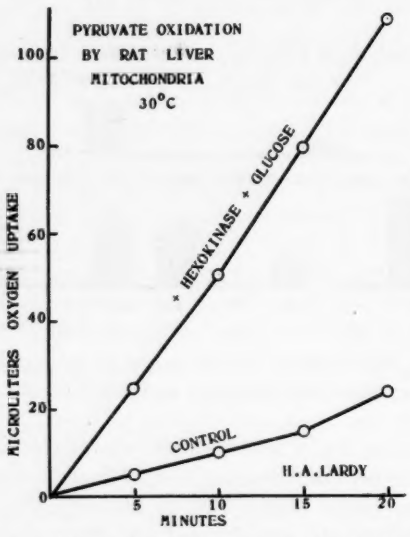

Fig. 4. STIMULATION of pyruvate oxidation by the presence of a phosphate acceptor system (31).

The experiments with isolated mitochondria, studied separately and in combination with other cell fractions or with a phosphate transferring system, provide models of the regulation of enzyme function in response to need, at the intracellular level. While a beginning has been made in this field, we feel that more rigorously defined preparations of cell fractions are needed for all studies of this type and therefore we would like to devote the remainder of our time to a brief discussion of methods.

METHODS

In a recent review Borsook (38) stated that the incorporation of amino acids into proteins does not necessarily depend upon the direct participation of the nucleus. While this statement may be true, it was based upon observations with mitochondria and microsomes that were almost certainly not free of nuclear material, owing to the fact that the cell fractions were obtained from material that had been subjected to the blades of a Waring blendor for 1½ minutes. This device is not suitable for this type of study on the formed bodies that occur in cells because under the conditions employed it tends to disintegrate both nuclei and mitochondria with the result that fragments of each contaminate the subsequent fractions, as will be described. The criteria for identifying these cellular components are not arbitrary, and while we are constantly looking for better criteria, some progress has already been made, as Dr. Hogeboom has shown today. It is our purpose to stress the use of at least *two* different types of analytical measurements for checking the homogeneity of cell fractions. We generally express the measurements on each cell fraction in terms of a percentage of the value for the total tissue sample. The two measurements are chosen to characterize the desired component and the chief contaminant, respectively.

Consider the isolation of nuclei. It is quite certain by now that all of the DNA of the cell is in the nucleus, and that the nuclei are sedimented at relatively low speeds in the centrifuge as the preceding speakers have emphasized. There are two points to be stressed in connection with recovery of DNA, which is the first objective in nuclear separations. When glass or lucite homogenizers are employed the recovery of DNA in the nuclear fraction is routinely near 100 per cent (1). However when the Waring blendor is used, the nuclei are completely disintegrated within 30 seconds in a KCl medium, according to reports from D. E. Green's laboratory (39, 40). Direct analytical evidence for nuclear disintegration in these preparations was reported by Schneider and Potter (28) who found only 74 per cent of the tissue DNA in the sedimented fraction. That under these conditions nuclear fragments contaminate subsequent fractions is shown in a report by Keller (41) who reported the

isolation of 'microsome' fractions containing large quantities of DNA following the use of the blendor. The second point is that the analytical recovery of 100 per cent of the DNA in the nuclear fraction is obtained with the use of trichloroacetic acid as in the original Schneider technique (42). Dr. Recknagel recently found that when perchloric acid was used for the extraction (cf. 43) the cytoplasmic fraction gave a false test for DNA, which was easily recognized by taking the complete absorption spectrum.

The second objective in the evaluation of the nuclear fraction is to determine the extent of contamination by whole cells and by cytoplasmic fractions. This information is provided by the second measurement, which under ideal conditions would be an analysis for something completely absent from nuclei but abundant in cytoplasm. Here we have chosen the determination of succinoxidase activity. It cannot be claimed on the basis of available evidence that the succinoxidase system is completely absent from nuclei, but it is the best test that we have seen for the quantitative detection of cytoplasmic contamination of nuclear fractions. By the use of pestle homogenizers, isotonic sucrose containing no electrolyte, and a low gravitational force of 400 *g*, we have obtained liver nuclei containing only 4 per cent of the total succinoxidase in the homogenate. For lower levels of contamination, only a careful examination with the microscope can assure complete absence of mitochondria.

The effect of the centrifugation medium is demonstrated in the next slide (fig. 5) which shows the packed nuclei from one gram portions of cells from perfused rat livers. Nuclei in water or in sucrose of any molarity between 0.1 and 0.8 M occupy a large volume that is 5 or 6 times as great as the volume in isotonic KCl or in isotonic sucrose containing a small amount of electrolyte (0.01 M phosphate). In addition to the effect on the nuclear volume, the presence of the electrolyte increased the contamination by mitochondria as evidenced by the high succinoxidase content, which was 23 per cent of the total, and by the increased uptake of the mitochondrial stain, Janus Green B, as shown in the photograph.

We next turned our attention to a re-examination of procedures for the isolation of mitochondria, again using two types of measurements as a guide to homogeneity. For the present it has been assumed that succinoxidase activity can be used to characterize the mitochondria, while PNA is being used to characterize the principal contaminant (microsomes). It is not implied that mitochondria contain no PNA, but it appears that they contain much less than the microsomes. Studies in the literature report rat liver mitochondria with PNA contents ranging from 5 to 40 per cent of the cellular PNA in normal rats on similar diets. The reason for this tremendous variation lies in the fact that the 'poorly sedimented' layer described by Schneider (1) has been removed by some investigators and left with the residue by others. It may be of interest to show a picture of this layer, and to show how a new application of the Janus Green stain has been employed by Dr. Recknagel to differentiate the two layers. This stain is considered to be specific for mitochondria (44) and it has also

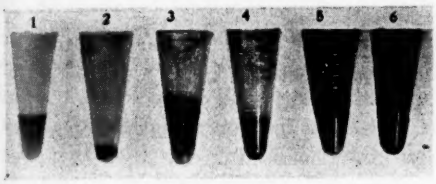

Fig. 5. EFFECT OF CENTRIFUGATION MEDIUM on volume of the nuclear fraction. Each tube contains the nuclei from 1 gm. of rat liver. Suspension media were as follows: *Tube 1*, H_2O; *tube 2*, isotonic KCl; *tube 3*, isotonic sucrose; *tube 4*, isotonic sucrose plus .01M potassium phosphate buffer, pH 7.6; *tube 5*, isotonic sucrose plus 50 γ Janus Green B; *tube 6*, isotonic sucrose plus .01M potassium phosphate buffer, pH 7.6, plus 50 γ Janus Green B. Centrifugation at 400 $\times$ *g* for 10 minutes.

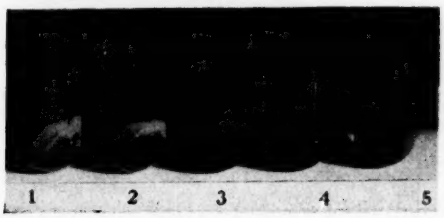

Fig. 6. DIFFERENTIAL STAINING of mitochondrial and poorly sedimented layers. Centrifugation at 8000 $\times$ *g* for 10 minutes following removal of nuclei. *Tube 1*, unwashed particulate matter; *tube 2*, washed once; *tube 3*, 30 γ Janus Green B added; *tube 4*, 100 γ Janus Green B, then incubated at 38° C. for 5 minutes. *Tube 5* shows incubated microsomes following addition of Janus Green B. The dark portion of the precipitate shown in *tube 4* was red.

been known that the mitochondria reduce the dye to diethylsafranin, which is red (44). It was found that the differential staining could best be developed by including the dye in the medium prior to centrifugation and by incubating the packed particles at 38° for 5 minutes. In the presence of an excess of the dye, both the mitochondria and the poorly sedimented layer originally stain blue, but upon incubation only the mitochondria turn red so that a sharp line can be seen between the two layers (fig. 6). The staining reaction and the reduction are optimum in isotonic sucrose, almost obliterated in 0.6 to 0.8 M sucrose, and weakened in hypotonic sucrose. In anaerobic suspension the reduction was stimulated by TPN but not by DPN.

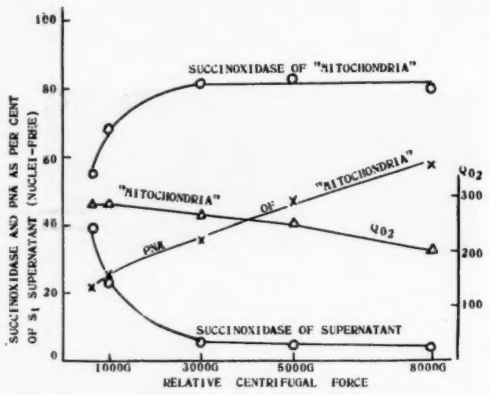

Fig. 7. DISTRIBUTION of liver PNA and succinoxidase activity at different centrifugal forces. The medium was 0.25M sucrose containing 0.01M phosphate pH 7.6. The poorly sedimented layer was not removed from the mitochondrial layer.

The next slide (fig. 7) shows succinoxidase and PNA measurements on the residues obtained at different centrifugal speeds, with the poorly sedimented layer left with the residue. It is clear that the succinoxidase recovery reached a maximum at relatively low speeds, while the PNA content and the amount of the poorly sedimented layer (observed visually) continued to rise as the gravitational force was increased.

On the basis of these findings, we concur in the opinion of Schneider and Hogeboom (1) that the particles in the poorly sedimented layer, at least in the case of liver, are probably not mitochondria, and that they are probably microsomes. Further studies on the microsomes and on other tissues using these and other criteria of homogeneity are needed.

The data in figure 7 may be compared with data employing the Waring blendor in which it was reported (40) that after 30 seconds in the blendor 45 per cent of the succinoxidase was in the 'microsome' fraction, and by 60 seconds the proportion occurring in the 'microsomes' had increased to as much as 73 per cent. *Less* than 30 seconds was insufficient time to break up the tissue. We interpret these data to mean that the blendor is unsuitable for cytochemical studies involving the methods of differential centrifugation as a prelude to studies on enzyme *systems* since it comminutes both the nuclei and the mitochondria to the point where they occur in the microsome and supernatant fractions. Further evidence regarding the increased phosphatase activity (45) and decreased oxidative capacity (46) of the blendorized preparations has been reported in other laboratories. We object to the use of the word 'homogenate' for these blendor preparations because it is clear that their properties differ considerably from the preparations to which we originally applied the word. The possible usefulness of the word homogenate has been destroyed by its indiscriminate use so that it can no longer be used without qualification. However, it was never an appropriate term for what is actually a *hetero*geneous suspension of cellular components, and its misuse is not too serious. The misuse of the words nuclei, mitochondria and microsomes is much more regrettable, although all of us are probably guilty in varying degrees. Moreover, the avoidance of the terms is undesirable if the data are to be useful in a cytochemical sense. The homogenate that we have used was merely a stage in the evolution of techniques for the study of enzymes at the intermediate level between the whole cell and the soluble protein (6). At this level, we feel that an effort should be made to relate enzymology to intracellular physiology, by maintaining the formed bodies of the cell in intact form insofar as may be possible, and by working with the separate components of the homogenate alone and in *defined* combinations.

The experiments we have described have been carried out with isotonic sucrose and with liver and kidney. We are advised that this medium may be unsuitable for tissues such as thymus and it should be emphasized that the methodology in this field is far from established.

SUMMARY

This discussion may best be concluded with a plea for more rigorously defined cell fractions and more careful definition of speeds, media, and homogenates. We predict that further attempts to isolate cell components with a minimum of post-mortem changes and to duplicate the intracellular environment will add greatly to our knowledge of intracellular control mechanisms. We believe that the understanding of these control mechanisms is our most important goal, because with such understanding comes increased power to cope with the problems of disease.

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STRUCTURE AND FUNCTION OF NUCLEIC ACIDS AS CELL CONSTITUENTS

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IT IS safe to say that living systems require the presence of both types of nucleic acid, or, in the case of parasitic systems, the presence of at least one. If, to use a designation of Schrödinger (1), one refers to the chromosomes as "the hereditary code-script," the great biological importance of all components of these nuclear structures, viz. nucleic acids, proteins, and, perhaps, lipids, is obvious, unless one assumes that one or the other of these components has been added by Nature as a meaningless and purely decorative flourish. This is, however, not likely.

In animals and higher plants the desoxypentose nucleic acids (DNA) are exclusively or almost exclusively situated in the nucleus. Pentose nucleic acids (PNA) are present in the nucleoli and the various cytoplasmic elements, e.g. the mitochondria, submicroscopic particles, etc. Recent work with Elson (2) on the nucleotide composition of PNA in different fractions of rat liver cells has provided preliminary evidence of differences in composition between nuclear and cytoplasmic PNA. It is not yet known whether this will be generally true; but results, recently reported with Magasanik (3), seem to point to the presence in pig liver PNA of two differently composed fractions.

Whether DNA really is limited to the nucleus is not entirely certain, since the available cytochemical or cytophysical methods presumably require the presence of a compact mass of DNA and may not reveal its occurrence in a diffusely distributed form, as could be the case in the cytoplasm of egg cells (cf. 4). The bacterial cell represents a special case. Whether the microbial nucleus is the sole repository of DNA in microorganisms, cannot yet be decided with certainty.

BIOLOGICAL SIGNIFICANCE OF NUCLEIC ACIDS

The discussion of the biological significance of a ubiquitous cell constituent is, strictly speaking, superfluous. But there exist a few important instances pointing to a direct involvement of nucleic acids; and some of them will be listed here briefly.

All virus preparations so far described are, or contain, nucleoproteins (cf. the recent survey of Davidson, 5). The same seems to be true of intracellular parasites, such as rickettsiae (6, 7) or paramecin (8).

Specific DNA preparations are known which are able to induce the transformation of bacterial types. This extremely important phenomenon, first discovered in pneumococci (9), has later been shown to operate also in *B. coli* (10) and in *Hemophilus influenzae* (11; cf. also 12). The possibility that reactions of this kind are of more general biological importance and not limited to the field of bacterial transformations cannot be rejected. What appears particularly remarkable is that it is here the free nucleic acid and not a nucleoprotein (as in the case of viruses) that is able to impose its own synthesis on the receptor cell, whereas in general nucleic acids seem to occur in cells only in the form of conjugated nucleoproteins. The mechanisms through which these transformations take place and the chemical features distinguishing these biologically active DNA specimens are completely obscure. There is, however, little doubt that it is the bacterial DNA itself, or a particular DNA fraction present in the transforming preparations, which is the carrier of activity. Recent work on the agent operative in the transformation of *H. influenzae* has shown that highly purified DNA preparations from two types are active in extremely low concentrations: 0.0004 γ of DNA per cc. in *type b;* 0.01 γ in *type c* (13).

The investigation of the relative efficiencies of different wave lengths of ultraviolet light has yielded curves closely resembling, but not entirely identical with, the UV absorption spectrum of nucleic acids. This work, mainly due to Stadler and Uber, Hollaender and Emmons, and Knapp and Schreiber, has been reviewed by Lea (14).

A certain degree of constancy, within the same species, of the DNA concentration per

diploid nucleus, and of roughly one-half this amount per haploid sperm nucleus, has been discovered by Boivin and his collaborators (15, 16, 4) and confirmed in other laboratories (17, 18).

DNA is in its composition identical in different tissues of the same species (19). Moreover, in the few cases where comparison was possible, no chemical differences have been observed between the composition of the DNA from the sperm cells and from differentiated tissues of the same species, in contrast to the very different composition of nuclear proteins in such instances.

DESOXYPENTOSE NUCLEIC ACIDS

All DNA preparations that have been studied in detail have several features in common. They are asymmetric molecules of high molecular weight (around 10^6), yielding extremely viscous solutions. They appear to contain the same desoxy sugar, namely 2-desoxyribose. They contain two purines, adenine and guanine, and two or three pyrimidines, viz. thymine and cytosine, and in several cases (20) also 5-methylcytosine. The desoxyribonucleotides released enzymatically from calf thymus DNA appear to be the 5-phosphates (21, 22).[1]

The conclusions to which our work has led us have been summarized recently (19, 24) and I shall limit myself here only to the main points.

1. DNA is in its composition characteristic of the species from which it is derived. This can in many, but not in all, cases be demonstrated by determining the ratios in which the individual purines and pyrimidines occur. There will, however, be very many borderline cases in which such differences in composition are not sufficiently significant to permit their use as the sole criterion of differentiation. The most important question of the sequence in which, in a particular nucleic acid, the nucleotides follow each other has so far barely been approached. The elaboration of methods for sequence analysis is, perhaps, one of the most urgent problems in nucleic acid chemistry, since differences in nucleotide sequence may very well be among the determinants of chemical and biological specificity.

2. No differences in composition have so far been found in DNA from different tissues of the same species. This provisional conclusion refers only to the over-all composition. It is in this connection noteworthy that no chemical differences appear to exist between the composition of DNA from normal human tissue (25) and that of preparations from human cancer tissue (26).

3. The tetranucleotide hypothesis is incorrect.

4. There exist a number of regularities. Whether these are merely accidental cannot yet be decided. In almost all DNA preparations studied until now the ratio of total purines to total pyrimidines never was far from 1. Similarly the ratios of adenine to thymine and of guanine to cytosine were near 1.

5. There appear to exist two main groups of DNA, namely the 'AT type,' in which adenine and thymine predominate, and the 'GC type,' in which guanine and cytosine are the major constituents. The latter has so far been found only in certain microorganisms (27).

Data to support these conclusions have, in the main, been presented in previous publications and were summarized recently (19, 24). I should like to limit myself here to the discussion of a few as yet unpublished results. The study of the composition of the DNA of salmon sperm (28) has brought out some of the regularities mentioned before particularly clearly (tables 1 and 2). This substance belongs to the 'AT type'.

Another investigation, undertaken in collaboration with G. Brawerman, deals with the DNA of wheat germ and the course of its degradation by crystalline pancreatic desoxyribonuclease. Previous work in our laboratory on the enzymatic disintegration of calf thymus DNA (29) had shown it to proceed according to a peculiar and complex pattern. The action of the enzyme resulted in the formation of dialyzable fragments and of a dialysis residue (core). The latter was

[1] In this connection mention may be made of experiments with D. Elson on the separation of the desoxyribonucleotides by filter paper chromatography. With neutral aqueous solutions of the nucleotides and the techniques described for the ribonucleotides (23) quantitative separation in the form of compact spots was obtained. With the buffered isobutyric acid-ammonium isobutyrate system (pH 3.6) the nucleotides were, in the order of increasing distance from the starting point, aligned as follows (with the distance of the adenine nucleotide arbitrarily taken as 100 and the relative distances of the other nucleotides placed in parentheses): *1*) Desoxyguanylic acid (53); *2*) thymidylic acid (66); *3*) desoxycytidylic acid (80); *4*) desoxyadenylic acid (100); *5*) desoxy-5-methylcytidylic acid (137). We are indebted to Dr. W. E. Cohn for the nucleotides.

characterized by greatly increased ratios of adenine to guanine, thymine to cytosine, purines to pyrimidines, and by greater resistance to enzymatic attack. Preliminary results of studies of this type on wheat germ DNA are presented in table 3. The analytical findings on the intact DNA are in good agreement with analyses recently reported for the same nucleic acid from

TABLE 1. SALMON SPERM DNA; PROPORTIONS (IN MOLES OF NITROGENOUS CONSTITUENT PER MOLE OF P IN HYDROLYSATE)

CONSTITUENT	MEAN PROPORTION	STANDARD ERROR
Adenine	0.280	0.005
Guanine	0.196	0.004
Cytosine	0.192	0.006
Thymine	0.274	0.005

TABLE 2. SALMON SPERM DNA; MOLAR RELATIONSHIPS

	MOLAR RATIO
Adenine to guanine	1.43
Thymine to cytosine	1.43
Adenine to thymine	1.02
Guanine to cytosine	1.02
Purines to pyrimidines	1.02
P accounted for as percentage of P in hydrolysate	95.8 (±1.6)
Average no. of gm.-atoms N per mole constituent	3.7
Atomic N:P ratio in DNA preparations	3.6, 3.7

two other laboratories (30, 31). The figures given here for the '19% core' and the '8% core' refer to the dialysis residues recovered when 81 and 92 per cent of the DNA respectively had been converted by the enzyme to dialyzable fragments. The trend of degradation appears similar to that observed with calf thymus DNA.

The molar ratios found in the DNA specimens studied in our laboratory are compared in table 4. The tendency toward certain regularities will be observed. The figures for hen DNA (chicken erythrocytes) and for the DNA from the K-12 strain of *B. coli* must be considered as preliminary. Both components were studied in collaboration with B. Gandelman. The DNA of *Hemophilus influenzae, type c*, was studied by S. Zamenhof. A few points are noteworthy. In the case of wheat germ DNA, methylcytosine and cytosine apparently must be considered together, if the regular ratios, observed in most other instances, are to be obtained. Another remarkable fact is that the regularities are maintained even in the nucleic acids of the 'GC type' despite the complete inversion in individual ratios.

As was already mentioned before, it is almost impossible to decide at present whether these regularities are entirely fortuitous or whether they reflect the existence in all DNA preparations of certain common structural principles, irrespective of far-reaching differences in their individual composition and the absence of an easily recognizable periodicity. It may be assumed that the nucleic acids as we know them today, as for that matter also the proteins, are the result of an age-long selection process in the course of which many less suitable or less stable components must have been eliminated. One could speak of the survival of the fittest nucleic acids. Such macromolecules will exhibit diversity and uniformity at the same time, since they are called upon to perform, in diverse species, the same tasks. It is, therefore, perhaps not astonishing that the nucleic acids will share certain features that may be directly connected with their stability or their ability to form conjugated nucleoproteins. One property which is quite striking is the uniform absorption spectrum in the ultraviolet of all highly polymerized DNA specimens, both with respect to the position and the intensity of the

TABLE 3. WHEAT GERM DNA; INTACT PREPARATION AND ENZYMATICALLY PRODUCED CORES (IN MOLES OF NITROGENOUS CONSTITUENT PER MOLE OF P IN HYDROLYSATE)

CONSTITUENT	INTACT DNA	19% CORE	8 % CORE
Adenine	0.27	0.33	0.35
Guanine	0.22	0.20	0.20
Cytosine	0.16	0.12	0.10
5-Methylcytosine	0.06	0.04	0.04
Thymine	0.27	0.26	0.23
Total purines	0.49	0.53	0.55
Total pyrimidines	0.49	0.42	0.37
Recovery	0.98	0.95	0.92

absorption maximum. The center of absorption fluctuates only between 257 and 261 mμ and the $(\epsilon)P$ is around 6600. Another surprising feature is the balance between amino groups and enolic hydroxyls in all DNA preparations examined by us. (See last column of table 4.) Even in the core preparations, this ratio changed only very little.

But let us return for a moment to the other outstanding characteristic of nucleic acids, viz. their diversity. If we accept the evidence of the existence of species-specific DNA, then there arise many new questions, both of a biological and chemical nature. DNA presumably is an important part of the chromosomes and may be surmised to be involved in their biological functions. Does this mean that a cell contains as many different DNA individuals as it contains genes? Or can one and the same species-specific DNA form so many three-dimensional structures, in connection with the proteins to which it is attached, that the genic requirements are fulfilled? (For a more detailed discussion of some of these points, cf. 32.) This question, as so many others in this field, cannot yet be answered. No way has as yet been found to fractionate a family of very similar macromolecules which may differ in no more than the sequence of a few of their component nucleotides. But one could perhaps say that the more regular the arrangement of nucleotides is in a given DNA, the less the chance of its forming many different specific structures. And this brings us again to the very important question of nucleotide sequence and periodicity in a nucleic acid chain. Any simplified assumption with respect to periodicity has been disproved by the studies on the course of action of desoxyribonuclease on the DNA of calf thymus (29) and of wheat germ. The composition of both the dialyzable degradation products and the dialysis residues, the 'cores,' exhibited continuous and characteristic changes with respect to the distribution of purines and pyrimidines. One must conclude that the sequence is highly aperiodic and that it is not inconceivable that the same cellular DNA could give rise to many different nucleoproteins, depending upon the shape and configuration of the particular protein.

TABLE 4. MOLAR RATIOS IN DNA PREPARATIONS OF DIFFERENT ORIGIN

SOURCE	ADENINE TO GUANINE	THYMINE TO CYTOSINE	ADENINE TO THYMINE	GUANINE TO CYTOSINE	PURINES TO PYRIMIDINES	AMINO GROUPS TO ENOLIC HYDROXYLS
Ox	1.29	1.43	1.04	1.00	1.1	1.4
Man	1.56	1.75	1.00	1.00	1.0	1.3
Hen	1.45	1.29	1.06	0.91	0.99	1.5
Salmon	1.43	1.43	1.02	1.02	1.02	1.4
Wheat	1.22	1.18[1]	1.00	0.97[1]	0.99	1.4
Yeast	1.67	1.92	1.03	1.20	1.0	1.3
Hemophilus influenzae, type C	1.74	1.54	1.07	0.91	1.0	1.5
B. coli K-12	1.05	0.95	1.09	0.99	1.0	1.6
Avian tubercle bacillus	0.4	0.4	1.09	1.08	1.1	1.7
Serratia marcescens	0.7	0.7	0.95	0.86	0.9	1.6
Hydrogen organism Bacillus Schatz	0.7	0.6	1.12	0.89	1.0	1.7

[1] In these computations the sum of cytosine and methylcytosine was used. If cytosine alone is considered, the thymine to cytosine ratio is 1.62 and that of guanine to cytosine 1.33.

It must, moreover, be understood that the recognition of periodicity, i.e. the presence of recurring units, will be particularly difficult in a macromolecule of the type of DNA. If, for instance, in a chain composed of 3000 nucleotides a particular sequence of 100 consecutive nucleotides were repeated 30 times, this periodicity could not be recognized, unless we had a method producing cleavage only at the points where these repeating units are joined. In other words, the perception of periodicity would require the proper distance for a bird's-eye view which will not be easy to attain.

Another approach to the problem of sequence analysis in DNA may be seen in the study of its controlled chemical degradation. That the purines can be detached from a nucleic acid with much greater ease than can the pyrimidines, has long been known; but the resulting end product,

thymic acid, was rather nondescript. In collaboration with M. E. Hodes and C. Tamm it has been possible to develop procedures, soon to be published, in which all purines could be cleaved from a DNA preparation, leaving behind a nondialyzable product in a yield of about 94 per cent that retained all the pyrimidines of the original DNA in unchanged proportions. We have designated preparations of this type as apurinic acid. These compounds may prove of interest for structural studies on DNA, since the position in the DNA chain of the initially present purine nucleotides is now marked by reactive aldehydo groups. The properties of a typical preparation of apurinic acid are contrasted in table 5 with those of the calf thymus DNA specimen that served as the starting material.

TABLE 5. APURINIC ACID FROM CALF THYMUS DNA

	DNA	APURINIC ACID
N	14.9	6.0
P	9.1	10.7
N/P	3.6	1.2
Absorption maximum, mμ[1]	258.5	268
Absorption maximum, ε(P)	6600	4600
Optical rotation[2]	+102°	+50°
Adenine, moles per mole P	0.28	0
Guanine, moles per mole P	0.21	0
Cytosine, moles per mole P	0.21	0.18
Thymine, moles per mole P	0.26	0.24
Thymine to cytosine	1.3	1.3

[1] In M phosphate buffer, pH 7.1. [2] In 0.1 M phosphate buffer, pH 7.1.

PENTOSE NUCLEIC ACIDS

Time does not permit an adequate discussion of the chemistry of PNA. In the past few years, significant contributions to this field were made by Gulland, Kerr, Schmidt, Allen, Loring, Cohn, and many other workers. I should like to make brief mention of some recent work done by B. Magasanik in our laboratory (3) in which the course of action of crystalline ribonuclease on PNA from yeast and from pig liver was studied with the use of chromatographic and spectroscopic procedures. About 60 to 70 per cent, or in some cases somewhat more, of the nucleotides present in the initial substrates were liberated by enzymatic action as rapidly dialyzable nucleotides of low molecular weight. This fraction consisted of free cytidylic and uridylic acids, which comprised a high proportion of the total pyrimidine nucleotides, and of combined purine and pyrimidine nucleotides. All PNA preparations yielded a nondialyzable residue, resistant to enzymatic attack, which was found to consist to about two thirds of guanylic acid and of varying amounts of the other nucleotides. A selection of these experiments is presented in table 6. These and other findings have led us to certain conceptions concerning the specificity of ribonuclease and the structure of PNA; but reference must be made to a detailed article which is in press (3).

TABLE 6. ACTION OF RIBONUCLEASE ON PNA

SOURCE	FRACTION	NUCLEOTIDE COMPOSITION (AS MOLES/100 M NUCLEOTIDE IN STARTING MATERIAL)				
		Guanylic acid	Adenylic acid	Cytidylic acid	Uridylic acid	Total
Yeast	Starting	30	21	23	26	100
	Dialyzable (total nucleotides)	18	15	19	26	78
	Dialyzable (mononucleotides)	0	0	14	19	33
	Core	66	18	8	8	
Pig liver	Starting	36	19	29	16	100
	Dialyzable (total nucleotides)	18	14	26	15	73
	Dialyzable (mononucleotides)	0	0	18	10	28
	Core	71	15	0	14	

FINAL REMARKS

It is fitting to conclude this all too sketchy survey with a confession of ignorance. What our studies have taught us more than anything else is how little we know as yet about the chemistry of nucleic acids. The chemical specificity of macromolecules and the interactions between them through which the organization of the cell is maintained can only partly be understood in

terms of our present knowledge. In the approach to a scientific problem two principles are operative: generalization and simplification. Both are necessary and both dangerous. It is obvious that we can learn more geometry from the illustrations in a textbook on projective geometry than from the beautiful pictures in Sir D'Arcy Thompson's *On Growth and Form*. But it is difficult to say where the danger line lies beyond which oversimplification will produce a dogmatic ignorance. Should we stress the multiformity of Nature, which makes us forget the simplicity of its basic designs; or should the essential shape win over the accidental form? In Wycherley's *The Country Wife* a quack is addressed as follows: "Doctor, thou wilt never make a good chemist, thou art so incredulous and impatient." If patience and credulity were all the chemist needed, the problem of the nucleic acids—still so baffling and elusive—would have been solved a long time ago.

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AMERICAN INSTITUTE OF NUTRITION

Symposium on Utilization of Protein

Chairman: A. H. SMITH

INFLUENCE OF NON-PROTEIN CALORIES ON PROTEIN METABOLISM[1]

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THE PRESENCE of energy-producing foods in the diets of all peoples reflects a primary metabolic demand. Sources of energy must be provided for the work of the body—intrinsic and extrinsic —for the maintenance of body heat, and for the regulation of the myriad biochemical reactions governing the physiological economy. There is, however, an accumulating body of evidence indicating that both carbohydrate and fat play a role over and beyond the provision of calories. That the total energy balance and the nitrogen balance are inextricably interwoven is evident. However, a review of the information found between the covers of that repository of fact and interpretation, *The Biology of Human Starvation* (1), indicates that, in respect to the three basic nutrients, carbohydrate, fat and protein, the requirements of man as we meet him in his ordinary walks of life have not been defined clearly in terms of practical working recommendations that will take into account the many and varied situations in which he finds himself. As in the past, knowledge of the nutritional requirements of human beings will grow as facts accumulated in research laboratories of this and other countries are interpreted and applied. I have chosen, therefore, today to confine myself largely to the presentation of experimental studies that may have significance in solving the many problems that are met in the feeding of the human race, the world over, in times of peace and war.

The story of the dietary interrelation of proteins, carbohydrates, and fats in the maintenance of nutrition had its beginnings in the laboratories of the great masters of physiology in Europe. As we, with our large fund of new nutrition knowledge and with research facilities of the highest order, look back to their achievements, our wonder ever grows that they could have accomplished so much. And certainly it is a challenge to the modern nutritionist to pick up the threads where these great men left off and to weave them into a finished tapestry.

Nitrogen balance is a tool that has been used by many workers in estimating the part played by non-protein calories in the establishment of nutritional state. I think we can say, in the main, that experimental studies show that nitrogen retention is a function in part of the caloric intake. However, there are many factors whose influence is not yet fully understood or appreciated that affect the metabolic interplay of these nutrients. What some of these factors are and how they work will be the theme of this discussion.

CALORIC RESTRICTION

It would seem that an understanding of caloric influence on the *endogenous* nitrogen metabolism characteristic of protein starvation should provide a base for fundamental first approximations. In figure 1*A* is shown the effect of systematic reduction of calories on the catabolism of 4 groups of rats as measured by the quantity of nitrogen excreted in the urine (2). The animals in each group had been protein-depleted under a standardized set of conditions and were catabolizing body tissue at a constant rate. The basal diet they received was protein-free, but otherwise adequate. When consumed *ad libitum*,

[1] Journal Paper, No. J-1958, Iowa Agricultural Experiment Station, Project 995.

it provided approximately 49 Cal./rat/day. In this experiment, the ration was fed in quantities that supplied 100, 75, 50, or 25 per cent of the calories normally consumed by the rat. The data indicate that the first two reductions in caloric intake although inducing some increments in urinary nitrogen do not impose stresses to which the animals cannot make physiologic adaptation. It is only when the energy intake is reduced to one-fourth of the normal requirement that the deficit becomes critical and the animals no longer are able to maintain the integrity of their tissues. The increment in the output of urinary nitrogen is immediate and marked just as soon as the caloric deficit is imposed.

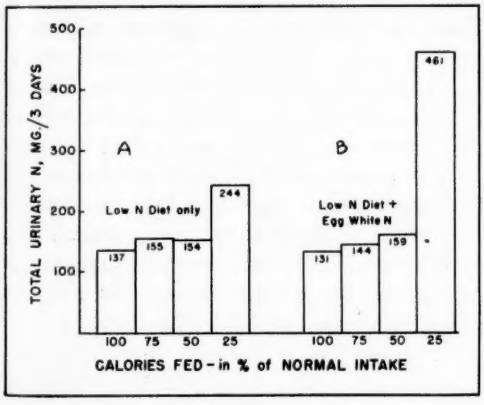

Fig. 1. URINARY EXCRETION of nitrogen by rats fed rations of different caloric values.

The way a protein-starved animal uses tissue protein under conditions of caloric restriction is reflected in its handling of protein derived from *exogenous* sources (fig. 1*B*). To the diets of the animals in this group, egg white protein was added in a quantity that supported nitrogen equilibrium when the ration *was of adequate energy value*. It may be seen that there is a trend toward an increased excretion of nitrogen in the urine as the energy value of the diet drops to one-half of the normal intake. On the whole, the average rates of metabolism of the rats in each group are not very different than they were when the diet contained no nitrogen. But further reduction in caloric intake leaves a marked imprint, and the animal throws all of the nitrogen of its food protein away so that it may use the non-nitrogenous portion as well as similar moieties arising from the degradation of body tissue in satisfying its demand for energy. The increment in urinary nitrogen when the diet supplies only one-fourth of the needed calories is approximately equal to the quantity of nitrogen ingested.

PHYSIOLOGICAL STATE

The experiments just described are based on the response of rats undernourished in respect to protein. Of as great interest is the influence of the non-protein calories of the diet on the manner in which animals better nourished in this respect utilize protein provided in their food, a subject studied in detail by Dr. Allison's group at Rutgers University (3). Again, when dietary protein is administered—this time to the dog—in a quantity that supports nitrogen equilibrium at full caloric intake, there is a continuously augmented excretion of nitrogen in the urine as the energy value of the diet drops from a high to a low value. Relative increments are of a somewhat greater order than they are in standardized protein-starved rats. However, as early as 1946, Allison and his co-workers showed that despite these constantly increasing urinary losses the nitrogen balance index of the dietary protein fed is not altered until the energy provided by the ration is less than one-half the normal requirement of the animal (4).

When the diet supplies more than 50 per cent of the needed calories, the internal reserve that an animal in a reasonably good state of nutrition possesses in its hepatic and plasma proteins acts like a safety valve. These proteins when drawn upon in times of emergency may provide sufficient energy to satisfy caloric needs and thereby permit utilization of food nitrogen. If the period of stress is not too prolonged, the animal apparently suffers no injury over and beyond the diminution of tissue reserves for meeting future dietary crises.

The picture in general supports the views of Bosshardt and his co-workers (5) that an animal responds in two ways to a systematic reduction of the caloric value of the diet. A nominal limitation causes the animal to draw on existing reserves to get the energy its food is not supplying; then more drastic deprivation forces it to use both food and body nitrogen for energy purposes, and a marked breakdown of body tissue occurs.

BODY STORES

The significance and importance of these body stores of nitrogen have been stressed by the Rutgers group (3). In animals possessing such

tissue reserves, metabolism under conditions of reduced caloric intake does not remain static, but rather drifts into the state where nitrogen retention is markedly reduced. The speed at which the drift occurs and the character of the metabolic response depend on the size of the internal stores of protein. A dog, for example, with well-filled tissue reservoirs passes first into acute negative balance when a severe caloric restriction is imposed. Then it drifts toward nitrogen equilibrium, but before this state can be achieved, the impact of the caloric deficiency forces the animal into acute negative balance. On the other hand, with the first restriction of calories, an animal whose initial reserves are low in protein slips immediately into positive balance, but then suddenly and rather dramatically drops into negative balance—perhaps a full 25 days earlier than does the better nourished dog. These experiments point to the fact that situations exist where an animal may be in nitrogen balance but still losing body tissue. The function of tissue reserves in padding metabolic processes against the shock of dietary imbalance may explain in part why Keys and his co-workers (1) did not observe a trend toward a correlation of nitrogen retention and caloric intake until the men had lived on the semi-starvation diet for more than 12 weeks.

RESISTANCE TO CALORIC RESISTANCE

It is interesting also to note that Allison (3) sometimes encounters a marked resistance to the nitrogen-depleting effect of a restricted caloric intake in individual dogs. Some of these animals are able to utilize dietary protein at full efficiency for as long a time as 150 days. Exploration of the reason will yield information, I am sure, that will enlarge our comprehension of the general problem.

QUANTITY OF PROTEIN IN THE DIET

The quantity of protein in the diet also bears a major relation to the response that an experimental animal may make to caloric additions or restrictions. Increasingly higher planes of nitrogenous utilization are established in mice consuming constant quantities of protein by additive quantities of calories. These, however, are enhanced further by increments in the dietary protein. It has been found in human experiments (6, 7) that, while 1500 Calories will support retention on a low protein intake—approximately the critical level—900 Calories are ample when the protein intake is doubled. On the other hand, we see in the 'Minnesota experiment' that in rehabilitation additions of protein to rations ranging in energy value from 2000 to 3000 Calories per day exert no favorable influence on the retention of nitrogen (1). It indeed will be of both theoretical and practical interest to learn the lowest combination of protein and non-protein calories that will sustain nitrogen equilibrium under a variety of conditions.

CALORIES AND THE 'TIME FACTOR'

The effect of the elapse of time that may occur between the feeding of the protein and carbohydrate moieties of the diet on the utilization of nitrogen has intrigued several groups of investigators for some time. There is Geiger's recent contribution (8), that in protein-depleted as well as in infantile rats (9), separate feeding of the carbohydrate and protein portions of the diet results in a diminished retention of nitrogen. In explanation, Geiger suggests that the simultaneous presence of carbohydrate and protein in the diet induces a continuous flow of the prerequisite essential and non-essential amino acids at a rate that is commensurate with the metabolic demands of repleting or growing tissue (10). Undoubtedly, Dr. Geiger will discuss the interesting phenomenon today. Indeed, his concept of the gastro-intestinal dynamics involved (11) may be a basis for explaining the extraordinary demand for calories in the attainment of nitrogen balance by young men ingesting pure amino acids and ammonium salts as their sole source of dietary protein (12).

In support of Geiger's views is Leverton's observation (13) of the protective influence of additional calories in alleviating the negative nitrogen balance induced in young women subjects by the exclusion of animal protein from one meal (14). This response occurs when the daily intake of protein is approximately 43 gm/person/day. Of interest is the fact that failure to include animal protein in each of the day's three meals is not associated with negative nitrogen balance when the daily intake of protein is raised to 63 gm., and that, with the inclusion of this much protein in the diet, the caloric intake may be as low as 1400 Cal/day with no adverse effects (15). So, quantity of dietary protein, the non-protein calories of the diet, the time factor, and the relative distribution of high quality protein in the

day's meal are intertwined together in the complex picture of protein utilization.

DIETARY SOURCES OF NON-PROTEIN CALORIES

Fat is regarded often as being wholly interchangeable with carbohydrate on a metabolizable energy basis. Also, it is generally assumed that the protein-sparing effect of carbohydrate is considerably greater than that of fat when subjects ingest the same quantity of protein. Furthermore, in complete protein starvation, it is believed that it is carbohydrate, and not fat, that has the property of sparing body protein (16). Under conditions of caloric restriction, however, we have found in our laboratory that fat exerts a profound body-sparing effect when included in protein-free diets fed to rats. Indeed, the data suggest that fat has unique properties in regulating the course of the protein and perhaps the energy metabolism (17).

In these experiments, as in those previously described from this laboratory, the catabolism characteristic of protein-starved rats on a full caloric diet serves as the reference point in studying the influence of dietary modifications—in this case, the inclusion or omission of dietary fat in daily food quotas of descending caloric value. Male rats, 6 months old, are brought to an approximately steady state of nitrogen metabolism as judged by the quantity of the element excreted in the urine (fig. 2). This condition with our particular stock animals prevails following maintenance for 18 days on a protein-free but otherwise adequate diet, and continues for another 14 to 16 days. All metabolic observations are made within this last interval of time. Two series of animals are prepared thus for study, the first group receiving a high fat ration during the period of depletion, the second a low fat diet.

The nitrogen retention of all animals in an experimental series, when consuming a ration poor in nitrogen but of full caloric value, is determined in a balance test beginning with the 19th day and lasting either 5 or 7 days. Then part of the animals are fed nitrogen-poor diets of reduced caloric value. Four days are allowed for dietary adjustments, after which nitrogen balance is determined again over an interval comparable in length of that of *period 1*. The remaining animals in the experimental series are continued on the full calorie diet and their balances redetermined.

Thus, the performance of the rats in any experimental group is controlled in two ways: i.e. by their own behavior in *period 1*, and by the performance of another set of animals catabolizing body tissue at the rate they would have been doing had no dietary modification been imposed.

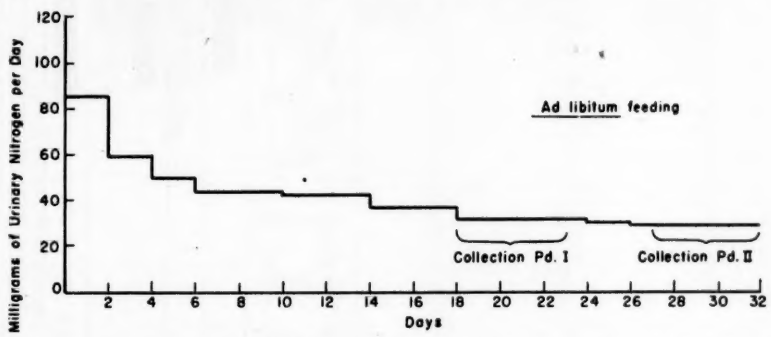

FIG. 2. EXCRETION OF NITROGEN during successive intervals by rats fed a nitrogen-low diet.

The basal high fat diet used contains 10 per cent each of lard and butter fat, 38 per cent of the calories coming from fat (table 1). Fifty mg. of vegetable oil supplement each diet. In the first experiments to be presented, the rations were offered *ad libitum* in *period 1*, and then held at this level of consumption in *period 2*. The rats so adjusted their intake of food that the average caloric value of the daily food quota of either the high fat or the low fat diet was approximately the same, i.e. 49 Calories. The vitamin mixture supplementing these basal diets contained all the recognized essentials.

In figure 3 are shown the nitrogen balances in *period 2* of rats fed the two basal diets—high fat and low fat—at four levels of caloric intake. The similarity in the rates of catabolism at normal and three-fourths normal caloric intakes suggests that the rats in the low fat group are not penalized

by the omission of the nutrient. Reducing the calories below this point, however, results in an intensification of the metabolic processes. Rats fed 12 Calories of the low fat diet, for example, lose tissue equivalent to 410 mg/nitrogen/100 gm. body weight in 7 days; those given the high fat diet, 266. The findings have some counterpart in the observations of Pearson and Panzer (18), who report that the loss of the essential amino acids in the urine and stools of rats is considerably reduced when corn oil is a dietary component.

TABLE 1. COMPOSITION OF BASAL DIETS

DIETARY COMPONENTS	HIGH FAT DIET	LOW FAT DIET
	gm.	*gm.*
Dextrin	73	93
Lard	10	0
Butterfat	10	0
O & M salts	4	4
NaCl	1	1
Ruffex	2	2
Total	100	100
Cal/gm	4.8	3.8
Av. intake in Cal/day	49 (18 rats)	49 (36 rats)

In another series of experiments in which food intakes were controlled on the basis of *ad libitium* ingestion in *period 1*, the effect of varying the fat content in the diet was investigated (fig. 4). The rations, isocaloric in value as fed, contained 20, 15, 10, 5 and 0 per cent of the butter fat-lard combination and were offered in quantities equivalent to 100, 75, 50, and 25 per cent of the normal energy requirement. The data depicted in the figure indicate that the nitrogenous metabolism of the animals is not altered until the dietary fat is reduced by 10 per cent; then the animals given the diet containing one-fourth the normal number of Calories go into a tailspin. The same condition prevails when the fat component is reduced further. In order to simplify comparisons, the nitrogen balances of all the groups whose caloric intake was held at one-fourth normal have been collected in figure 5. Seemingly, a ration should contain at least 15 per cent fat if it is to exert a protective action against the disintegration of body tissue induced by severe caloric restriction in a protein-starved rat.

We have obtained considerable evidence that fats originating from different plant and animal sources vary in their capacity to protect against the body catabolism characteristic of the present experimental situation. Discussion of the relative merits of different fats does not seem warranted until results have been replicated and confirmed. Some related problems have been discussed recently by Barki, Collins, Elvehjem and Hart (19).

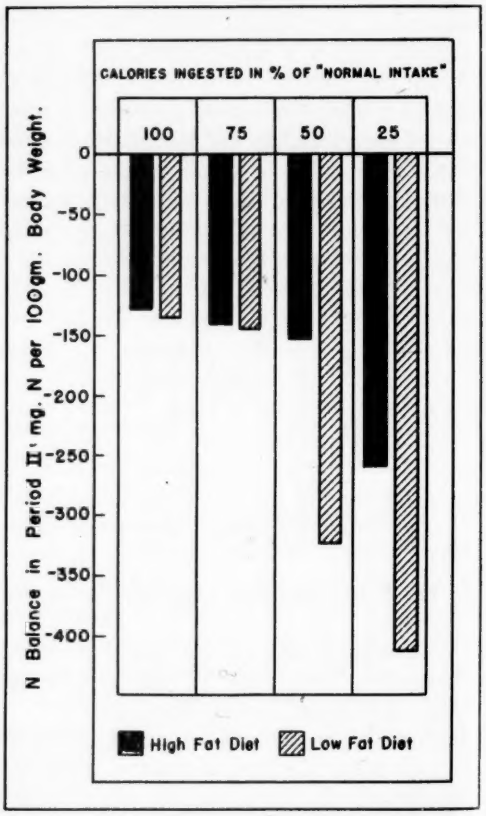

Fig. 3. NITROGEN BALANCES induced by feeding protein-free diets high and low in fat and of graded caloric value.

Inasmuch as rats fed rations deficient in protein involuntarily restrict their consumption of food, it seemed important to rule out the possible influence of inadequate food intake by more rigid control of this factor. Hoover, therefore, working in our laboratory, studied the response of animals to the force feeding of protein-free diets providing 100 per cent and 25 per cent of the daily energy requirement (20). The rations were made into slurries of the same caloric density, and the portions of each administered daily provided either 56 or 14 Calories/300-gm.

rat (fig. 6). Again, the presence or absence of fat in the diet is not reflected in any change in the total nitrogen balance when the animals are administered food supplying the full quota of necessary energy. Forced feeding, however, dramatically aggravates the rate of catabolism when only 14 Calories are given. Negative balance in these rats now is 1465 mg/300-gm. rat/5 days, in contrast to 514 in the restricted group of fat-fed rats given the same number of calories in the form of the high fat diet.

inadequate flood of nutrients from the gastrointestinal tract.

Nitrogen balance data presented thus far suggest that if the animal receives its full asset of calories, the removal of fat from the protein-free diet inflicts no handicap in the animal's adjustment to the absence of dietary protein. But before this conclusion is reached, let us examine other data. It was noted, for example, during the course of various force feeding experiments that a number of rats always died, and that this

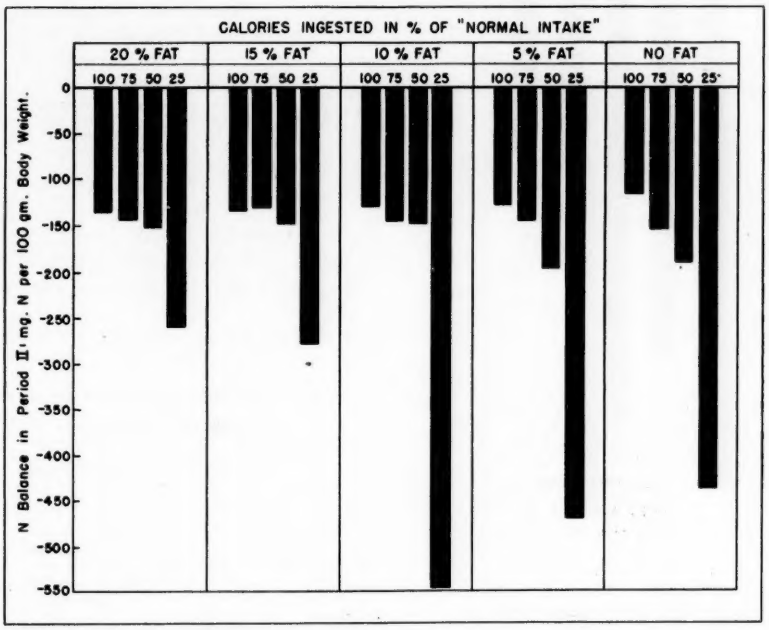

FIG. 4. NITROGEN BALANCES induced by feeding protein-free rations of varying fat content and of graded caloric value.

A comparison of the data recorded in table 2 would indicate that when *ad libitum* feeding is employed, deprivation of calories in the form of both carbohydrate and fat increases the catabolism about 1.8 times; in the form of carbohydrate alone, 3 times. On the other hand, when force feeding is employed, the latter ratio becomes 5.

Why force feeding aggravates the unfavorable response to the ingestion of the low fat diet is a moot question. It should be noted that the controlled feeding regime reduces variation within a group. Perhaps these data indicate that the loss of appetite associated with the ingestion of inadequate diets represents a protective physiological mechanism against metabolic obstacles encountered in the handling of a qualitatively

mortality occurred in the group receiving the full intake of the low fat diet (table 3). In the first 3 experiments listed, mortality in the group ranged from 30 to 50 per cent, whereas none succumbed in the groups fed the high fat ration. The response of *group IV* shows the effect of offering the daily quota of food in three feedings rather than two, and of equalizing the densities of the food slurries by the addition of ruffex to the high fat diet. It must be of significance that of the 207 rats observed deaths occurred only among the groups fed the fat-poor diet. Also it is noteworthy that no rats in either group maintained on restricted caloric intake died.

I am wondering here if we may not have evidence of an acute fat deficiency disease precipitated by the strain of metabolic adjustment to

a protein-free diet. Decker and his co-workers (21) have written recently that chronic essential fatty acid deficiency may culminate as an acute syndrome by the imposition of various stresses and strains.

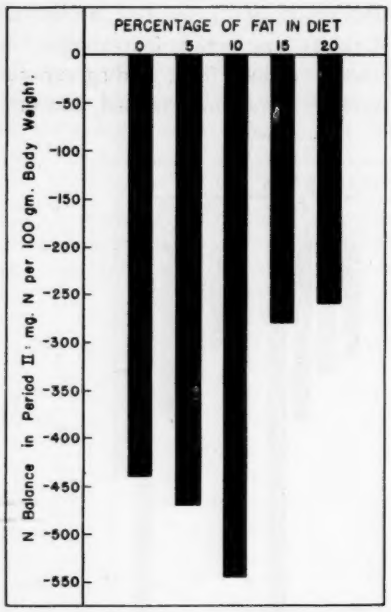

Fig. 5. NITROGEN BALANCES when rats are fed protein-free diets of varying fat content and supplying only one fourth the needed calories.

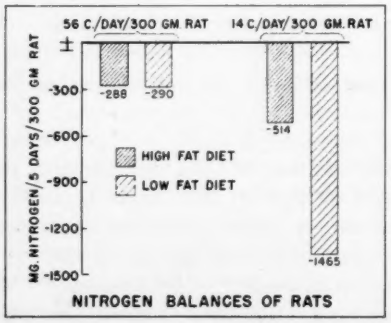

Fig. 6. NITROGEN BALANCES induced by the force feeding of protein-free diets high and low in fat at two caloric levels.

In studying further the effect of the removal of dietary fat, determinations of the partition of nitrogen in the urine (fig. 7) disclosed an interesting alteration in the pathway of nitrogen metabolism when carbohydrate in restricted quantities served as the sole source of energy. It is not possible to present all data today, but it should be indicated that a complete partition was made, and that in every experimental group the sum of the nitrogen present in the various components equalled the total excretion of nitrogen.

Restricted caloric feeding induces a high excretion of urea in the fat-fed group. The increment is over two and one-half times greater, however, in the group receiving the low fat diet. But of greatest interest is the extremely large excretion of ammonia nitrogen in the urines of this group of rats.

TABLE 2. FIVE-DAY NITROGEN BALANCES WHEN CARBOHYDRATE CALORIES REPLACE FAT CALORIES IN THE DIET

REPLICATIONS OF EXPERIMENT	METHOD OF FEEDING	LOW FAT DIET		HIGH FAT DIET	
		Full caloric intake	Restricted caloric intake	Full caloric intake	Restricted caloric intake
year		*mg/300-gm. rat*	*mg/300-gm. rat*	*mg/300-gm. rat*	*mg/300-gm. rat*
1946	*Ad lib*[1]	−201	−724	−231	−418
1948	*Ad lib*[1]	−225	−725	−235	−412
1949	Force	−290	−1465	−288	−514

[1] In *period 1* and held at this intake in *period 2*.

TABLE 3. MORTALITY OF RATS FORCE-FED PROTEIN-FREE RATIONS

GROUP NO.	TIME	LOW FAT RATION		HIGH FAT RATION	
		No. of rats	No. of rats dying, %	No. of rats	No. of rats dying, %
		56 Cal./day/300-gm. rat		*56 Cal./day/300-gm. rat*	
I	Spring 1949	48	31	48	0
II	Summer 1949	27	48	12	0
III	Fall 1949	12	50	12	0
IV	Winter 1949	12[1]	25	12[1]	0
	Winter 1949	12	17	12[2]	0

[1] Three feedings per day. [2] Ruffex added.

Examination of these data, in connection with the concentrations of urea nitrogen and amino nitrogen in the blood, may be of interest (table 4). Feeding the low fat ration at full caloric intake results in an increment in the relative concentration of amino nitrogen in the blood over that in the blood of rats receiving adequate calories and fat in their ration. Some of this apparently spills over into the urine, and chromatographic determinations of amino acids therein disclose their presence in largest quantities in

the urines of rats fed unrestricted amounts of the low fat diet.

The response of the rats in the low calorie-low fat group permits interesting speculation. What initiates the tremendous outpouring of nitrogen when this ration is fed in the first instance, and why does the animal receiving fat in its diet seemingly do a better job in adjusting to the stress of caloric restriction in protein starvation than its carbohydrate-fed control? Perhaps the speeding up of the catabolic processes may reflect

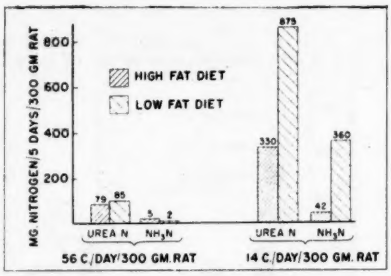

Fig. 7. UREA NITROGEN and ammonia nitrogen in urines of rats fed protein-free diets high and low in fat.

TABLE 4. CERTAIN NITROGENOUS CONSTITUENTS IN URINE AND BLOOD

ENERGY VALUE OF DIET PER 300-GM. RAT	RATION	URINE			BLOOD	
		Urea N	NH_3 N	$-NH_2$ N	Urea N	$-NH_2$ N
Calories/day		*mg.*	*mg.*	*mg.*	*mg. %*	*mg. %*
56	High fat	79	5	4	6	26
56	Low fat	85	2	12	2	47
14	High fat	330	42	9	6	34
14	Low fat	875	349	4	31	18

the increased specific dynamic effect of a simultaneously metabolizing pool of carbohydrate and protein in relation to one of fat and protein (22). But I do not believe that this is all of the answer.

Possibly, here, there is a block in the course of the carbohydrate metabolism. Normal rats on restricted caloric intakes and receiving the same quantity of nitrogen in their food protein as the rats in this experiment liberate from tissue sources can transform the nitrogen of their food amino acids into urea, thereby presumably deriving full energy value from the non-nitrogenous portion. The high proportion of ammonia nitrogen to urea nitrogen in the urine of a calorie-starved rat suggests that the animal, in the absence of fat, is unable to take acidic intermediaries arising in the catabolic freeing of amino acids through the metabolic transformations required for their utilization as energy, and the kidney is forced to manufacture ammonia to care for the excretion of these intermediaries.

There are other evidences of an impaired carbohydrate metabolism. Inability to convert the non-nitrogenous portions of the degraded protein molecule into glycogen is shown by an extremely low reserve of glycogen in the livers of the rats fed the restricted low fat diet, i.e. 0.7 per cent vs. 2.0 per cent in the fat-fed groups. The glucose tolerance curves of the four groups

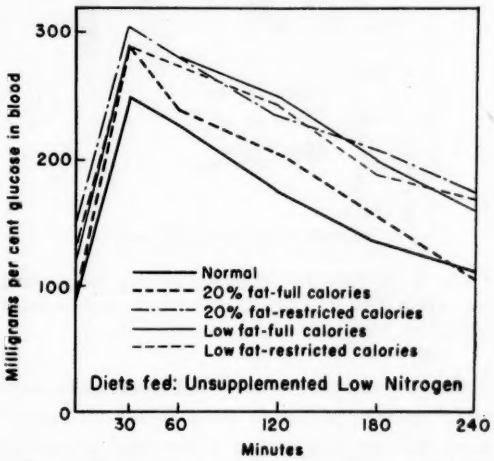

Fig. 8. GLUCOSE TOLERANCE curves of rats fed the stock ration and various modifications of a low nitrogen diet.

in relation to that of normal stock animals are also interesting in this connection (fig. 8). The tolerance of the rat receiving its full caloric quota of the high fat diet most nearly approaches the normal. Inanition may explain the lower tolerances of the rats fed restricted number of calories—either high fat or low fat—but it is significant that the average tolerance curve of the animals with a *luxus* consumption of carbohydrate calories falls in the same range as the tolerances of the rats restricted as to their caloric intakes. On the whole, the animals maintained on the low fat diets have many characteristics that are diabetic-like in nature. Others have noted that rats subsisting on a low fat diet cannot tolerate the increment in metabolic rate induced by the feeding of thyroid (23).

Very pertinent in this connection is the sug-

gestion from the work of Swift and his co-workers at the Pennsylvania State College, that rats fed diets containing fat use energy derived from dietary sources more efficiently than animals on a low fat dietary regime (22). In the words of these workers, the presence of fat in the diet confers "economy of utilization of food energy." Supporting this hypothesis that fat is needed for the maximal use of energy are the observations of several groups of workers showing that, under differing experimental conditions, inclusion of fat in the diet is associated with an increased capacity for work (24, 25).

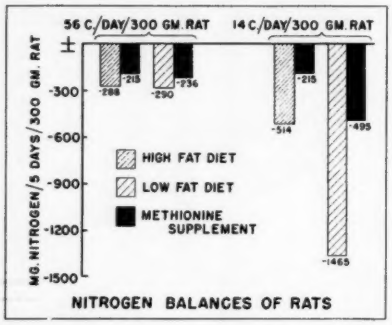

Fig. 9. NITROGEN BALANCES of rats fed protein-free diets high and low in fat when methionine is added as a dietary supplement.

Possibly our own observations that methionine seems to play a role in these metabolic conversions will provide a clue in the final unraveling of the problem (fig. 9).

Its addition to the protein-free diet in quantities equivalent to 4 mg/nitrogen/day results in an immediate reduction in the total nitrogen excreted by all experimental groups, but particularly by the low fat-low calorie rats, and thence to a decrease in the negativity of the nitrogen balance. The concentrations of urea nitrogen in the urines of both groups fed 14 Calories drop concomitantly (fig. 10). There is a fall in the excretion of ammonia when methionine supplements the low fat diet, but not when it is added to the rations of rats receiving the restricted amount of the high fat diet. That despite the marked decrement in total urinary nitrogen the relative proportion of ammonia to urea nitrogen in the urines of the rats fed 14 Calories of the low fat diet remains the same as it was before the methionine was administered, suggests that methionine plays its part in regulating the process, whatever it is, that catalyzes the catastrophic breakdown of body tissue.

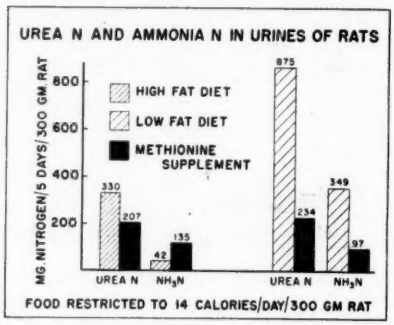

Fig. 10. EXCRETION of urea and ammonia by rats fed protein-free diets supplemented with methionine and providing 14 Cal/day/300-gm. rat.

On the basis of the discussion today, nitrogen retention represents the interaction of many factors, i.e. caloric value of the diet, source of non-protein calories in the ration, physiological state, body stores, individual resistance, quantity of dietary protein particularly in relation to the energy value of the diet, the time factor, and the distribution of animal protein over the day's meals. Only when the influence of these and probably other factors in the establishment of nutritional state in man is fully understood will it be possible to state his protein requirements in terms of practical recommendations.

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EXTRA CALORIC FUNCTION OF DIETARY COMPONENTS IN RELATION TO PROTEIN UTILIZATION[1]

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"Interpretations based on single factor relations do not adequately take into account the multipliciiy of other factors, both in the organism and in the experimental diet, which obviously condition the results of the experiment."

J. and TH. GILLMAN[2]

A NECESSARY requirement for the utilization of dietary proteins and amino acids in growth and maintenance is the availability of all essential nutrients in the body. Deficiencies of vitamins, minerals, or any other essential food elements usually result in cessation of growth, loss of weight and a negative nitrogen balance. Since all the 'essential' food elements are normally supplied by the diets, a discussion of the effect of dietary composition on amino acid utilization would comprise practically the whole territory of intermediary metabolism, which is beyond the scope of this paper.

We will rather discuss more specifically the effect of those food ingredients which are fed simultaneously or within a short interval of time before or after consumption of the proteins.

The sparing effect of dietary fat and carbohydrate on protein utilization was recognized early and was explained by the concept of *isodynamism*. The idea that dietary carbohydrate may have a specific effect on protein utilization in addition to its calorigenic action was often hinted in the early literature concerning the dietotherapy of diabetes. Rose (1), in his fundamental paper on essential amino acids, stressed as early as 1937 the point that, "factors such as the proportion of fat and carbohydrate in the ration . . . may play important roles in determining the minimum level of a given component," i.e., essential amino acid.

Since that time several investigators indicated the existence of a specific effect of dietary components on the utilization of amino acids and proteins.

In recent studies on the development and possible nutritional improvement of meal patterns, we investigated the importance of time relations in the consumption of different food ingredients. In confirmation of earlier experiments (2), we found that the nitrogen-sparing effect of carbohydrate depended on the time which had elapsed between the feeding of the protein moiety and the carbohydrate moiety of the diet. When sugar had been fed more than four hours before or after the protein meal, no nitrogen-sparing effect could be observed.

In well-fed *adult* rats, the nitrogen-sparing effect could be demonstrated during the first few days only, after which time the nitrogen balance, in spite of subsequent simultaneous feeding of sugar, returned to its original level, probably because of the inability of the adult organism to store abnormally high amounts of protein. Further investigations on *growing* rats as well as on *protein-depleted adult* animals in which increased and prolonged protein demand existed demonstrated that both the growth response in infant animals and the repletion in depleted adults was superior when sugar was given simultaneously with protein, as compared with control animals which received sugar and protein separately, the total caloric intake being identical in both instances. In these latter experiments the nitrogen-sparing action of sugar lasted not only for 2 to 3 days, but for the duration of the experiments, i.e., 14 to 21 days (3). These results indicate that dietary nitrogen-containing

[1] The unpublished experiments discussed in this paper were performed in collaboration with I. El Rawi, E. B. Hagerty and L. E. Geiger. The experiments have been supported by grants from the National Vitamin Foundation, the Williams Waterman Fund of the Research Corporation and by the Van Camp Sea Food Co., Inc.

[2] In *Human Malnutrition*. New York: Grune and Stratton, 1951.

compounds when fed simultaneously with carbohydrates are retained and utilized for growth and repletion, i.e., they do participate in normal protein metabolism.

Such simultaneously fed sugar or any other dietary ingredient may *a priori* influence the protein utilization at one or all of the three following phases: in the gastrointestinal phase, in the phase of body protein formation and in the metabolic phase.

in growth promotion, had to be investigated. Consequently, *adult* male rats were induced to eat spontaneously definite quantities of either skim milk or a standard casein powder or one of the strained meats. Two, four and six hours later the nitrogen in the stomach and intestinal content was determined. We were surprised to find that the milk protein disappeared much more slowly from the digestive tract than any of the other proteins tested. Our thinking has been

TABLE 1. GASTROINTESTINAL ABSORPTION OF NITROGEN-CONTAINING COMPOUNDS

MATERIAL FED	CONSUMED NITROGEN ABSORBED AFTER				RESIDUE AFTER TWO HOURS	
	2 hr.	4 hr.	6 hr.	8 hr.	In stomach	Intestines
	%	%	%	%	%	%
Skim milk, spray dried	18.3	36.9	59.3	79.2	91.5	8.5
Whole milk, spray dried	22.0	53.3	70.3		94.0	6.0
Fish[1]	55.7	71.2	92.2	93.6	80.4	19.6
Pork	36.5	63.6	85.6	92.0	79.7	20.3
Casein	56.6	81.3	99.0		86.0	14.0
Lactalbumin	31.8	66.7	83.8		60.0	40.0
Fish[1] plus 0.66 gm. glucose	27.4		79.0		69.3	30.7
Fish[1] plus 0.66 gm. cellulose	55.7				74.5	25.5
Casein plus 0.6 gm. glucose	37.5	73.1	99.0		81.0	19.0
Casein plus 0.8 gm. lactose	32.0				93.0	7.0
Casein plus 0.6 gm. cottonseed oil	15.8		54.6		78.0	22.0
Casein plus 0.6 gm. sucrose	43.0		97.0		88.5	11.5
Casein digest[2]	89.4	93.0			1.0	99.0
Casein digest[2] plus 0.6 gm. glucose	81.6				29.0	71.0
Casein digest[2] plus 0.4 gm. cottonseed oil	71.4				73.0	27.0
Zein	40.0		46.0		2.0	98.0
Gelatin	72.0				5.0	95.0

The different proteins were fed in quantities containing approximately 100 mg. nitrogen to adult male rats, after 24 hours' starvation. Experiments with casein, zein and gelatin indicate that gastric emptying time is dependent on some characteristics of the protein.

[1] Strained tuna.

[2] Spray dried acid hydrolysate fortified with tryptophan.

The importance of the gastrointestinal phase in this respect was suggested by some experiments in which the nutritive value of commercial 'strained' meat products was investigated, using as a standard spray-dried skim milk. We found that diets containing skim milk as the only protein source had the strongest growth-promoting effect on infantile rats; the next best effect was obtained with diets containing strained tuna; while strained beef, pork, veal and lamb proved to be less effective.

Since the amino acid inventory of these proteins is nearly identical, the possibility that some differences in the digestibility of the proteins used could account for the observed differences conditioned by the rather unsubstantiated claim that 'easily digested, easily absorbed' proteins are nutritionally superior to the others. Therefore, the result of this investigation, indicating that the protein source which promoted growth best disappeared most slowly from the digestive tract, was unexpected (see table 1).

We should consider, however, that the growth rate is determined by inherent regulatory factors and is not accelerated by superoptimal supply of the building stones. Tissue protein synthesis, and hence growth, requires the *simultaneous* presence of all the amino acids. Evidently the dietary proteins are best utilized for growth when the rate of supply from the digestive tract corre-

sponds closely with the growth requirements. When the rate is too fast, the utilization will suffer because no excess of amino acids can be stored normally for later use; the surplus is either excreted or irreversibly metabolized (4, 5). Therefore, a delay in the protein digestion and absorption should tend to improve the utilization. Such a mechanism is suggested also by the investigation of Leverton and Gram, as well as Wu and Wu (6, 7). Their experiments on adult human beings indicate that the utilization of dietary protein improves when intake and resorption are evenly distributed throughout the day by consuming more frequent protein meals.

We recently found that lactalbumin, which supports growth to a higher degree than casein, disappears more slowly from the intestinal tract than does casein. This observation seems to favor the outlined ideas.

Experiments prompted by such considerations revealed that simultaneous consumption of sugar does delay the disappearance of protein and absorption of its cleavage products from the digestive tract, suggesting that the delay in the intestinal resorption may well be involved in the nitrogen-sparing action of sugar fed simultaneously.

Further experiments have shown that in accordance with the classic observations of Ewald and Boas (see 8) on gastrointestinal motility, simultaneously fed fat, even more than carbohydrate, considerably delayed the disappearance of protein from the digestive tract.

A similar effect of fat on resorption of another nutrient has been reported by Nief and Deuel (9), who found that the rate of intestinal absorption of galactose varies inversely with the percentage of fat present in the diet, and that fat secures a better utilization of this sugar by preventing 'galactose-flooding' of the organism.

Based on these results, the effect of simultaneous fat feeding on growth has been investigated. One group of infantile male rats received casein from 7 P.M. to 11 P.M. and a protein-free, but otherwise complete, diet from 7 A.M. to 3 P.M. Another group received the fat moiety of the diet mixed to the protein meal. It was found that the rats receiving fat with the protein grew markedly better than the controls, although the total caloric and protein intakes were identical (see fig. 1).

The proper balance between rate of supply and rate of utilization seems to be even more critical in feeding amino acid mixtures than in feeding protein. Accordingly, the growth-promoting action of carbohydrate and fat is more pronounced when fed simultaneously with a tryptophan supplemented casein hydrolysate.

The experiments discussed so far are in complete agreement with many earlier data. For example, it was reported that rats fed equicaloric diets grew better if the fat content of the diet was increased (10, 11). The protein digestion was improved; the nitrogen retention increased; and the fecal and urinary excretion of some essential amino acids, such as valine, lysine and methionine, was less on diets containing corn oil than on a fat-free diet.

These effects could be explained by the observation that simultaneously fed fat generally delays the intestinal passage and thus improves

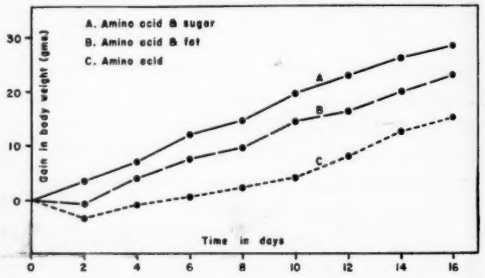

Fig. 1. EXPERIMENTS in which the protein moiety of the diet was fed either separately or together with sugar or fat. Infantile male rats of the Wistar strain were used. Each group contained 8 rats with a starting weight of 50 to 55 gm.

digestion of the protein and the absorption and utilization of amino acids.

This explanation is, however, at variance with the results of some other authors (12, 13), who found that an increase of the fat content of the diets which were kept isocaloric with the control diet promotes growth without affecting nitrogen retention.

Munro has shown, furthermore, that fat, unlike sugar, when fed simultaneously with protein, fails to increase nitrogen retention in *adult* rats. In view of our experiments with carbohydrate, we investigated the effect of simultaneously fed fat on the repletion of protein-*depleted* rats and found that, contrary to the effect of sugar, simultaneously fed fat did not increase nitrogen retention.

However, it should be emphasized that our results cannot be compared directly with those of some earlier authors since the daily fat intake in our experiments was not changed—the same

amount of fat was fed either apart from or together with the protein moiety of the diet.

We believe that in addition to its action on gastrointestinal resorption, fat also exerts an effect on protein utilization at some other level. It has been reported (10, 11) that animals kept on protein-free, isocaloric diets, lose less weight if the fat intake is kept high. Evidently the high fat intake decreases also the catabolic destruction of body tissue. Furthermore it was reported that the damaging effects of excessive methionine doses can be antagonized by increasing the fat content of the diet.

These experiments would indicate that although simultaneous fat intake improves protein utilization by retarding the rate of amino acid supply from the digestive tract, its total effect must be much more complex.

Returning to the protein-sparing action of carbohydrates, it was found that although the retardation of absorption is smaller than in the case of fat, the growth promotion and the nitrogen-sparing effect are much more pronounced when carbohydrate is fed simultaneously with protein instead of fat. This indicates that the effect on absorption is only a minor factor involved in the protein-sparing action of sugar.

Following the fate of food constituents after transfer from the digestive tract to the body proper, they may affect the utilization of the absorbed amino acids in the process of tissue protein synthesis. Since protein synthesis requires the simultaneous presence of all the constituent amino acids, the most obvious effect of sugar seems to be the provision of precursors for the formation of missing nonessential amino acids. This possibility was investigated in collaboration with Dr. Arne Wick (14) by feeding C^{14}-labeled sugar and ammonium phosphate to growing or protein-depleted rats, together with or apart from Rose's mixture of essential amino acids. It was to be expected that if the sugar is utilized for amino acid formation and thus supports the protein utilization, more labeled material will be taken up by newly formed or repleted tissue. The results obtained on normal adult rats do not substantiate this hypothesis, although the error of the procedure may still mask possible differences. Experiments are now in progress in which the uptake of labeled carbon by the protein formed during liver or plasma regeneration is investigated. These recent studies show that protein isolated from the regenerating liver tissue contains considerably more C^{14} when sugar and essential amino acids are fed together. This supports the hypothesis that simultaneously fed sugar promotes protein synthesis by providing the necessary precursors for the synthesis of nonessential amino acids.

Recently published experiments (15) in which more nitrogen was retained by the body if ammonium salts were fed together with sugar than without sugar also seem to indicate that simultaneously fed sugar may expedite the utilization of nitrogen used for amino acid synthesis.

The conditions are still more complicated in growth where the formation of new tissue requires probably, besides the necessary amino acids, also the simultaneous availability of other cell constituents. How far a fast-growing organism can draw on reserves of such factors as minerals, etc., if they are missing from the diet has not yet been investigated.

That some of the nutrients such as vitamins once deposited in the tissues are no longer easily available can be demonstrated by feeding to vitamin-depleted rats tissues of their equally depleted litter mates. The result is usually a dramatic improvement. We therefore investigated whether growth of rats is influenced when the water-soluble vitamins are fed simultaneously with or apart from the protein-containing diet and found that in some experiments the growth was definitely improved when the vitamins were fed simultaneously with the diet, while in others there was no apparent difference. However, the rats receiving the vitamins separately from the diet never grew better than the others. For all practical purposes the growth was the same on meals containing vitamins or with later vitamin supplementation.

That the feeding of some minerals in proper concentration may improve the utilization of dietary proteins has been recently proposed by McQuarrie and Ziegler (16). These authors reported that by balancing the phosphorus and calcium content of the diet the biological value of meat products can be raised to that of milk. We repeated the latter experiments, adjusting the calcium and phosphorus content of all the diets to values equal to the milk diet. In spite of this, the efficacy of the diets was the same as in the control experiments. The growth on milk was best; then strained tuna, beef, followed in order (fig. 2).

We are still at a loss to explain the reason for the differences between the results of the Minneapolis group and ourselves. However, our experi-

ments show that equalizing the P, Ca, Mg and K content in itself does not raise the biological value of the meat diets to that of the diet containing milk protein.

How the intake of precursors or of substances promoting the formation of cell constituents such as lipids, certain carbohydrates and nucleoproteins influences the amino acid utilization has yet to be investigated. One perhaps may object that the organism with intact lipo- and glyconeogenetic faculties is able to form such compounds itself and does not depend on dietary supply. Such objections usually result from a misinterpretation of Schoenheimer's fundamental concept of 'dynamic equilibrium' and from the indiscriminate use of the term 'metabolic pool.' It has been, therefore, assumed erroneously that it is irrelevant whether substances like sugar have been produced in the intermediary metabolism or were provided with the diet. In the first place, it is questionable whether or not such substances produced in the body can be supplied at a rate optimal for tissue synthesis.

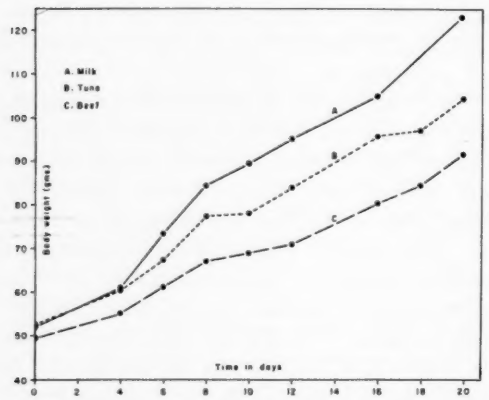

Fig. 2. GROWTH EXPERIMENTS with diets containing equal amounts of calcium, phosphorus, magnesium and potassium. Each group contained 6 male rats of the Long Evans strain.

Evidently a substance carried in high concentration by the portal system from the digestive tract to the liver has a different effect and fate than when produced in the tissues. For instance, absorption of dietary sugar increases the insulin production and also activates the anterior pituitary gland (17, 18). It has been shown that both hormones improve protein utilization (19). We investigated whether such increased hormone production could be held responsible for the nitrogen-sparing effect of simultaneously fed sugar. In these experiments diabetic as well as hypophysectomized animals reacted like normal rats so that the Langerhans islands and the anterior pituitary play no major role in the nitrogen-sparing action of sugar.

The third point at which dietary constituents may exert their influence on protein utilization is the 'metabolic phase,' comprising formation, destruction and transformation of amino acids.

A specific action of fat on the utilization of cystine was reported by Salmon (20) who also found that the nicotinic acid requirement is decreased in fat-rich diets. This latter effect may spare some tryptophan and divert it to other metabolic pathways.

The problem in which we are particularly interested at the present time is how sugar may exert its action on amino acid utilization in this metabolic phase. One possibility under investigation is that the simultaneous resorption of sugar protects the circulating amino acids from premature deamination in the liver. This possibility is supported by the experiments of Krebs (21) who found that the deamination by liver slices *in vitro* is decreased in the presence of easily oxydizable material such as lactate, pyruvate, etc. The decreased specific dynamic action of protein when fed simultaneously with sugar also suggests protection against catabolic changes.

It is likewise possible that some preliminary steps for protein synthesis, such as acetylation or phosphorylation is promoted by simultaneously fed sugar. This hypothesis is supported by Miller's (22) experiments in which it was found that the uptake of labeled lysine during liver perfusion is promoted by sugar.

SUMMARY

We tried to demonstrate that dietary substances fed together with proteins do promote growth and utilization of the dietary amino acids, quite apart from their calorigenic sparing action. Of course, some of our conclusions are still open to objections and will have to be supported by further experiments. Especially, the effect of parenteral administration of fat and sugar will have to be investigated.

We believe that besides the effect on intermediary metabolism, special attention should be given to the effect of food ingredients on gastrointestinal dynamics, including motility, gastric emptying time, as well as the effect on the production of bile and other digestive juices

and, finally, on their influence on rate of digestion and absorption.

We have discussed mainly factors which promote protein utilization but it is known that some ingredients such as cocoa (23) may interfere with protein digestion and with the efficiency of its utilization.

The possibility should not be overlooked either that some effect may be produced by the food ingredients tested indirectly by influencing the bacterial flora (24).

We did not discuss the mutual effect of different dietary proteins and amino acids on their utilization, although the phenomena of supplementation and amino acid imbalance may also be influenced by the presence of other factors in the meal.

It is unknown how far the reported findings on animals are applicable to human nutrition. Therefore we refrain from drawing practical conclusions at the present time. We are convinced, however, that the indicated lines of study may eventually lead to a scientific planning of meal patterns with an optimum utilization of available protein resources.

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INTERPRETATION OF NITROGEN BALANCE DATA

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RECENTLY, Mitchell (1) has presented an excellent outline of protein metabolism which is based on Folin's (2) concept of two distinct types of metabolism but includes later developments, particularly those of Whipple (3) and Schoenheimer (4) and their associates. This outline accepts the principle that endogenous metabolism represents irreversible reactions involving tissue proteins and other nitrogenous constituents as typified by the dehydration of creatin to creatinine. Exogenous catabolism could be defined as catabolism of nitrogenous constituents in amounts exceeding the needs of the animal for endogenous maintenance and growth. Dietary and body nitrogen may be said to meet in a common metabolic pool of blood and tissue proteins, a pool that can be drawn upon for either exogenous or endogenous purposes. Exogenous catabolism could include, therefore, the utilization of dispensable stores of body proteins, stores which have been described as being raided for specific amino acids not supplied in the diet (3, 5, 6).

The depletion of body protein stores is reflected by a decrease in excretion of urinary nitrogen. When an animal is placed on a protein-free diet, urinary nitrogen excretion decreases, rapidly at first, then more and more slowly. This initial rapid loss of nitrogen represents the catabolism in part of labile protein stores. After these stores are depleted the loss of nitrogen is less rapid, urinary nitrogen excretion approaching a low and constant value (fig. 1). It is interesting to note that, during regeneration, accomplished by feeding protein, those nitrogen stores that are depleted last are probably refilled first. These results suggest that the stores depleted last are more essential and are replenished before those which are dynamic and dispensable (7).

A better understanding of nitrogen stores can be obtained, however, from nitrogen balance than from urinary nitrogen excretion. Nitrogen balance (B) is the difference between dietary nitrogen intake (I) and nitrogen excreted in the feces (F) and urine (U) so that:

$$B = I - (F + U) \qquad (1)$$

The animal is gaining nitrogen when B is positive, it is losing nitrogen when B is negative, and it is in nitrogen equilibrium when B is zero.

The relationship between nitrogen balance and nitrogen stores is illustrated in figure 2. These results were obtained by feeding alternately a protein-free diet and one containing protein to a dog (8). The white bars record the nitrogen balance during the protein-free period, while the bars with slanted lines represent the balance while the animal was receiving a constant nitrogen intake. The magnitude of the protein stores of this dog was average, resulting in a negative nitrogen balance of −2.4 gm. of nitrogen/day/m.^{2} of body surface area (first white bar). Protein was added to the diet, but not sufficient protein to put the dog in nitrogen equilibrium. Continued negative nitrogen balance resulted in further reduction in the catabolism of body stores so that, when the animal was returned to the protein-free diet (second white bar), the urinary nitrogen excretion was reduced still further and the nitrogen balance was less negative. Continuation of this type of alternate feeding resulted eventually in depletion of the protein stores to such an extent that the amount of nitrogen being fed produced a positive balance. Thus, nitrogen balance is a variable, changing with time as the protein stores are increased or decreased.

The relationship between nitrogen intake and balance is illustrated by the curves in figure 3. It is assumed that the data for these curves were determined over a short period of time, so that nitrogen balance was not altered significantly by continued feeding of nitrogen. *Curve A* was obtained by feeding whole egg protein to a dog depleted in protein stores. The low stores are demonstrated by the relatively low negative balance of −1 gm. of nitrogen/day/m.^{2} of body surface area. A small amount of dietary nitrogen was required to maintain nitrogen equilibrium in this animal. The curve extends far into the region of positive balance, showing marked capability on the part of the animal to fill depleted stores. A maximum was reached at approximately

+6 gm. of nitrogen/day/m.² surface area. *Curve C* illustrates data obtained while feeding the same protein to a dog with full protein stores. The fullness of the stores is reflected by the balance of −4 gm./day/m.² surface area and by the relatively large quantity of dietary nitrogen necessary to maintain nitrogen equilibrium. Since the stores of this dog are essentially full, only a slight positive nitrogen balance was produced. The degree of depletion of the stores in an animal can be estimated by the magnitude of positive balance which is produced by a given nitrogen intake.

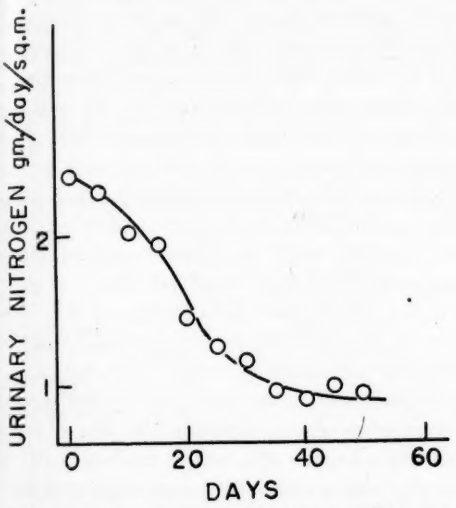

Fig. 1. URINARY NITROGEN EXCRETION (gm/day /m.² body surface area) in a dog fed a protein-free diet for 50 days.

Curves B and *D* represent data obtained while feeding wheat gluten instead of whole egg protein. The rate of increase of nitrogen balance with nitrogen intake was less with wheat gluten than with whole egg in the diet, and the maximum positive balance was also less. The nutritive value of the dietary proteins can be measured in terms of the slopes of the lines in figure 3, and by the maximum positive balance which can be produced.

The curves in figure 3 are essentially linear until the point of maximum utilization is reached. The relationship, therefore, between nitrogen balance (B) and absorbed nitrogen (I) can be expressed by the following equation:

$$B = KI + B_0 \qquad (2)$$

where K is the slope of the line and B_0 is the balance when the nitrogen intake is zero. This equation may be rewritten to eliminate nitrogen balance as follows:

$$U = (I-K)\,I + U_0 \qquad (3)$$

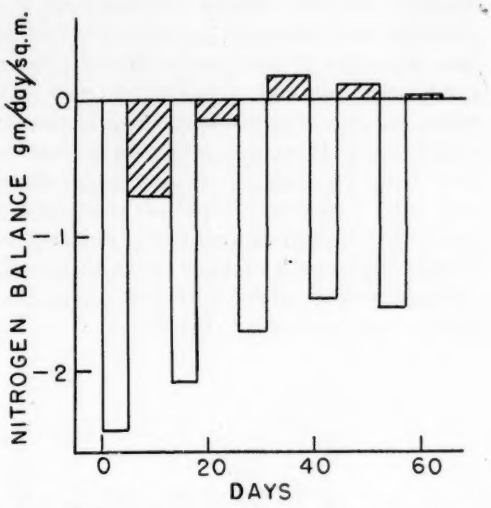

Fig. 2. NITROGEN BALANCE (gm/day/m.² body surface area) in a dog fed a protein-free diet (*white bars*) alternately with diet containing .09 gm. of egg white nitrogen/day/kg. body weight (*bars with slanted lines*).

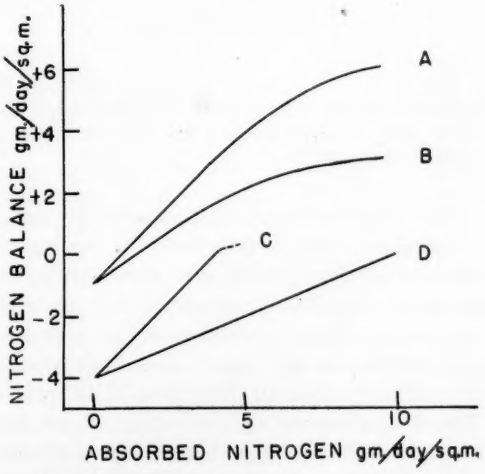

Fig. 3. NITROGEN BALANCE VS. absorbed nitrogen in a protein-depleted dog fed whole egg protein (A), in a protein-depleted dog fed wheat gluten (B), in a normal dog fed whole egg (C) and in a normal dog fed wheat gluten (D).

where U is the excretion of urinary nitrogen during nitrogen feeding and U_0 is the excretion of urinary nitrogen on a protein-free diet (9). It

has been proven (9, 10) that K in these equations is the fraction of absorbed nitrogen retained in the body of the animal, provided the excretion of body nitrogen (B_0 or U_0) is constant over the whole range of nitrogen intakes. The fraction of absorbed nitrogen retained in the body is by definition the 'biological value' (11) of the protein. The slope of *line D* is 0.4. If body nitrogen excretion is constant, then 40 per cent of the wheat gluten nitrogen was retained in the body of the animal. It was pointed out in the introduction, however, that dietary and body nitrogen may enter a common pool which is available for catabolism as well as anabolism. It is not surprising, therefore, to find evidence that feeding nitrogen may decrease or increase the contribution of body nitrogen to catabolism.

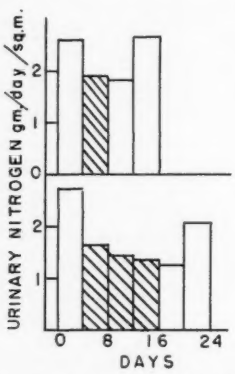

Fig. 4. AVERAGE DATA obtained on dogs fed protein-free diet (*white bars*) and protein-free diet plus 1 gm. of *dl*-methionine per day per dog (*bars with slanted lines*).

The conservation of body nitrogen by dietary nitrogen has been demonstrated by feeding methionine to dogs (12–14) and to rats (15). The excretion of urinary nitrogen in the dog is decreased by adding a small amount of methionine to a protein-free diet (fig. 4). Less body nitrogen is contributed to the catabolic pool in the presence than in the absence of methionine. It has been suggested (16) that the demands for methionine in the dog are relatively great so that body protein reserves are utilized to supply this amino acid, the excess catabolism of nitrogenous constituents thereby contributing to an increased excretion of urinary nitrogen. Mitchell (1) has suggested that the extra demand for methionine is the result of the growth of hair at the expense of other tissues of the body, the hair demanding large amounts of sulfur amino acids. Addition of an optimal amount of methionine to some proteins such as casein can increase the value of K in *equation 2* to values over unity. Values over unity would be impossible if B_0 in the equation represented the excretion of body nitrogen at all nitrogen intakes. Values over unity can be interpreted to mean that feeding nitrogen reduces catabolism of the nitrogenous constituents in the metabolic pool. Swanson and coworkers (14) have given several other examples of the nitrogen-sparing action of dietary nitrogen in the rat.

Dietary nitrogen may also increase the utilization of the nitrogen in the body pool. A large amount of methionine (5%) in a diet containing casein when fed to the rat increases the catabolism of these stores (17). The data suggest, too, that the metabolism of excess methionine requires the simultaneous catabolism of fat (18) and of the utilization of the amino acid glycine (19). Excess methionine may draw on the nitrogen pool for glycine, thereby causing increased catabolism of other amino acids in the pool. Addition of glycine together with methionine reduces this excess catabolism. The beneficial effects of glycine may be due to the participation of this amino acid in the catabolism of methionine, glycine being the precursor of serine. One pathway for the metabolism of methionine is by the way of a combination of homocysteine and serine to form cystathionine (20–22). Other examples of raiding of nitrogen stores have been suggested by the work of Whipple and associates (5, 6). They have found that blood proteins can take priority over other tissue proteins, that depleted dogs will continue to produce much plasma protein and hemoglobin for many weeks while being fed a low nitrogen diet. An inadequate protein-like globin, or an inadequate mixture of amino acids can result in formation of blood proteins, other tissue proteins possibly being depleted to supply the deficient amino acids (23).

Since K in *equations 2* and *3* need not be equal to 'biological value,' this constant has been called the nitrogen balance index of the dietary protein (24). The index is a function of the fraction of nitrogen retained in the body of the animal. It is more specifically the rate of change of nitrogen balance with respect to nitrogen intake, and it is a relative measure of the amount of nitrogen necessary to maintain equilibrium, a measure of the over-all utilization of nitrogen by the animal.

Table 1 records nitrogen balance indexes for six standard proteins or protein foods in adult dogs. These data were obtained from experiments which were a part of a cooperative study of protein evaluation sponsored by the Bureau of Biological Research of Rutgers University. The effects of supplementing casein with methionine and wheat gluten with lysine on the nitrogen balance indexes are illustrated in the table.

The index for whole egg protein in adult dogs is usually found to be equal to or better than egg white. The relatively low index for whole egg protein recorded in table 1 is believed to be associated with changes produced during processing and drying of this particular whole egg preparation. It is interesting to compare these indexes with some determined several years ago on different samples. These earlier determinations yielded the following indexes: casein plus methionine, 1.2; egg white, 1.0; beef, 0.85; casein, 0.80; and wheat gluten, 0.44 (8). The casein with the index of 0.80 was a cruder sample than the one with the index of 0.73 (table 1) and probably had a higher methionine content. The beef sample with the index of 0.85 was selected from the best cuts of meat while the sample listed in table 1 was representative of all types of beef protein. These indexes for beef in the adult dog are much higher than values reported by Morgan *et al.* (25), a difference which emphasizes the susceptibility of the nitrogen balance method to variations in technique and in the physiological state of the animal. Some of these variations will be mentioned in the following discussion.

TABLE 1. AVERAGE DATA OBTAINED ON SIX DOGS

PROTEIN SOURCE	ABSORBED NITROGEN I	URINARY NITROGEN U	PROTEIN-FREE URINARY NITROGEN U_0	NITROGEN BALANCE INDEX E
		gm/day/m.²		
Casein + methionine	3.3	2.02	2.60	1.17[1]
Egg white	1.79	1.69	1.94	1.14
Whole egg	1.73	2.53	2.30	0.87
Beef muscle	3.02	2.75	2.05	0.77
Casein	3.10	2.74	1.90	0.73
Wheat gluten + lysine	2.04	3.02	2.44	0.73(7)
Peanut flour	4.22	3.74	1.89	0.56
Wheat gluten	3.01	3.09	1.11	.044

The nitrogen balance indexes were calculated from *equation 3* in the text.

[1] Average on 3 dogs.

The index for raw egg white was found to be abnormally low in one so-called normal dog. This low value was associated with poor digestibility which could be attributed to the antitryptic factor in raw egg white. Poor digestibility of raw egg white was found also in most dogs depleted in protein stores (7). Morgan *et al.* (25, 26) have found the dog, particularly the puppy, to be vulnerable to this antitryptic activity of egg white.

Nitrogen balance indexes all tend to increase when the protein stores of the dog are depleted markedly. Indexes, for example, in protein-depleted dogs were found to be as follows: egg white, 1.12; whole egg, 1.06; casein, 0.84; and wheat gluten, 0.70 (7). In general, however, indexes are essentially constant and independent of the magnitude of the protein stores until those stores have been depleted markedly. Nitrogen balances, on the other hand, vary with time, the animal always drifting toward equilibrium if the balance is either negative or positive. In this way the stores are depleted or repleted to a point of equilibrium if the balance is either negative or positive. In a growing animal, fed an adequate protein the positive nitrogen balance per gram of nitrogen intake decreases rapidly as the animal approaches adulthood, a decrease which is illustrated in figure 5. Nitrogen balances per gram

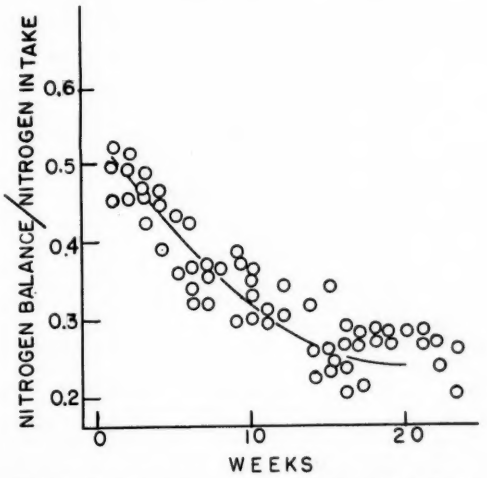

Fig. 5. NITROGEN BALANCE/nitrogen intake in puppies fed 25 per cent casein diet beginning at time of weaning (8 weeks).

of nitrogen intake have been found to have greater significance as measures of protein utilization in the growing dog than the customary protein efficiency (grams gain in weight/gram of nitrogen intake). The data in table 2 compare nitrogen balance with body weight gain data for the evaluation of dietary proteins in beagle puppies. These data were calculated over a period of several weeks of the fast-growing period of the animal.

TABLE 2. AVERAGE INCREASE IN BODY WEIGHT PER GRAM OF NITROGEN INTAKE B. W./I AND AVERAGE POSITIVE NITROGEN BALANCE PER GRAM OF NITROGEN INTAKE B/I IN THREE BEAGLE PUPPIES DURING FAST-GROWING PERIODS (15)

PROTEIN SOURCE	B. W. / I.	I. B. / I.
Whole egg	9.0	0.50
Casein	9.8	0.43
Wheat gluten	8.8	0.18

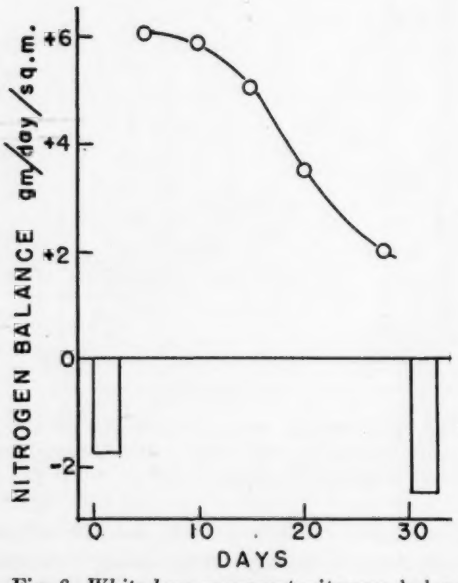

Fig. 6. *White bars* represent nitrogen balances in a dog fed a protein-free diet before and after repletion. *Circles* represent positive nitrogen balances produced while repleting with a constant intake of whole egg protein.

These data demonstrate that weight gains are not as accurate a measure of the utilization of a protein as nitrogen balance. The puppies fed wheat gluten increased their caloric intakes above those receiving whole egg. They became fat and soft, while those fed whole egg became lean and hard, but both sets of dogs weighed about the same after 70 days on the respective diets. Growth of the protein stores, however, was much greater in the dogs fed whole egg than in those receiving wheat gluten.

Positive nitrogen balances decrease in depleted adult dogs fed a constant nitrogen intake. This gradual drop in nitrogen balance is illustrated in figure 6. The first bar in negative balance records the excretion of nitrogen on a protein-free diet. The gradual drop in positive balance during the protein-feeding period can be interpreted to be a reflection of increasing protein stores. The increased catabolism of these stores is illustrated by the greater negative balance obtained while feeding a protein-free diet following the repletion period (second white bar). Similar experiments using wheat gluten have demonstrated that, even after prolonged feeding of this protein, the balance on a protein-free diet is often not made more negative, but may even be more positive. These results suggest that wheat gluten may deplete some protein stores to aid in the repletion of others (7).

The proteins of the body and nitrogen balance can be effected also by the caloric intake (27). Recent experiments in dogs demonstrate that the nitrogen balance decreases regularly as the caloric intake decreases. Data in table 3 were obtained on dogs receiving a constant nitrogen intake of 3.82 gm. of casein nitrogen/day/m.^{2} of body surface area. As the caloric intake was reduced, the nitrogen balance decreased from +0.68 to −0.74. Measurements of nitrogen balance were taken over a short period of time, so that the protein stores of the body were not markedly altered by prolonged feeding. Other experiments (26) demonstrated that the nitrogen balance index was not altered initially by the decrease in calories. Curves such as those illustrated in figure 3 were shifted downward farther into the region of negative balance but without altering their slopes. The values for B_0 in table 3 illustrate this shift into negative balance. These values were calculated using an average index of 0.73 for casein determined over short feeding periods for all caloric intakes except the very lowest of 95 calories/day/m^2. These results suggest that restriction in calories increases the catabolism of exogenous reserves of both body and dietary nitrogen. The increased catabolism, however, does not alter at first the mechanism of filling these stores as measured by the nitrogen balance index. When the more labile body stores are re-

duced, however, the index decreases rapidly, essential tissues are depleted, and irreversible damage may be done if the caloric restriction is continued. It has been demonstrated that animals with adequate body protein reserves can resist the depleting effects of a caloric restriction over long periods of time while those with inadequate stores rapidly deteriorate. The rapid deterioration is illustrated by *curve A* in figure 7. *Curve A* represents data obtained on a dog partially depleted in protein so that 3.82 gm. of casein nitrogen/day/m.^{2} and 480 calories/day/m.^{2} produced a positive nitrogen balance. In the absence of abundant labile protein stores, the index was immediately reduced and the animal drifted rapidly into negative balance. Nitrogen integrity of the body could not be maintained in this animal at a low caloric intake even with nitrogen in the diet. *Curve B* represents data obtained on another dog with large protein stores, so that 3.82 gm. of casein nitrogen/day/m.^{2} and 480 calories/day/m.^{2} were not sufficient to put this animal into positive balance. Prolonged feeding depleted these stores, the dog approached nitrogen equilibrium, but because of the low caloric intake eventually drifted into a period of rapid loss of nitrogen. Thus *dog B* resisted the damaging effects of caloric restriction over a longer period of time than *dog A*. Indeed, until *dog B* began to drift rapidly into negative balance, this animal was alert and seemed to be in good clinical condition. Loss of interest in daily routine, irritability and marked weakness was always associated with the drift into negative balance following a restriction in calories.

TABLE 3. AVERAGE VALUES FOR CALORIC INTAKE AND NITROGEN BALANCE (B) FOR THREE DOGS FED 3.82 GM. CASEIN NITROGEN/DAY/M.^{2} OF BODY SURFACE AREA

CALORIES/DAY/M.^{2}	NITROGEN BALANCE B GM/DAY/M.^{2}	PROTEIN-FREE NITROGEN BALANCE (CALCULATED) B_0 GM/DAY/M.^{2}
3190	+0.68	2.10
2860	+0.18	2.60
2290	+0.13	2.65
1910	−0.10	2.88
480	−0.22	3.00
95	−0.74	

Protein-free nitrogen balances (B_0) were calculated assuming an index of 0.73 (27).

It should be emphasized that these data were obtained on dogs being fed a low fat diet containing a quantity of casein which maintains the average dog on an adequate caloric intake in nitrogen equilibrium. The response to a caloric restriction is a function of the balance between dietary constituents as well as of the physiological state of the animal. Further evidence for the importance of the balance between calories and proteins is found in the work of Schwimmer and McGavack (28), who demonstrated that young men fed a low nitrogen intake (3 gm. egg white nitrogen/day) did not retain dietary nitrogen until the caloric intake had been increased to 1500 calories. If the intake was increased to 6 gm/day, the nitrogen was retained at a much lower caloric level (900 calories). Bosshardt *et al.* (29) demonstrated that an increase in calories at a constant protein level improved the utilization of protein, which was enhanced still further by the addition of more protein. The authors suggest that protein is the most important limiting factor in many states of semi-starvation.

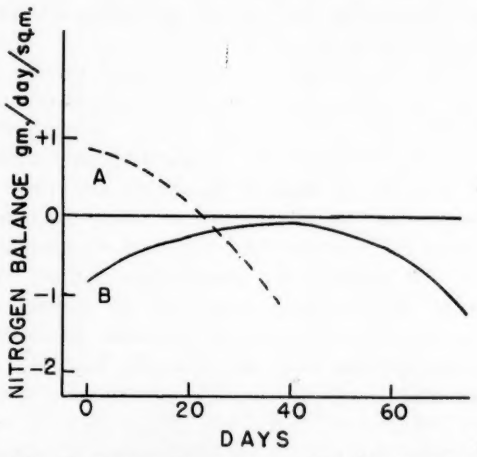

Fig. 7. NITROGEN BALANCE (gm/day/m.^{2}) vs. days of feeding a reduced caloric intake (480 calories/day/m.^{2}) and a constant nitrogen intake of 3.82 gm. casein nitrogen/day/m.^{2}. *Dog A* was partially depleted in protein stores at the beginning of the experiment; *dog B* had adequate stores.

Data in the literature suggest that fat in the diet exerts a role over and beyond the provision of calories, possibly through an effect on protein metabolism. Swanson and collaborators (30),

studying the effect of nitrogen excretion in low nitrogen diets, reported that "the catabolism of rats, ingesting isocaloric quantities of high fat and low fat rations, proceeded at essentially the same rate. However, upon adjustment of the energy value of the diet to one-fourth of the customary caloric consumption, the elimination of fat doubled the destruction of body tissue." Schwimmer and McGavack (28) found that a diet of 900 calories, containing 30 per cent fat and 6.0 gm. of nitrogen daily, decreased the urinary excretion of nitrogen in men below the excretion when the diets provided 10 and 20 per cent fat. They believed that "the nitrogen-sparing effect of 30 per cent fat was not due to increased calories, but rather to something intrinsic in the higher fat per se."

The balance between protein and calories is very important for the maintenance of normal tissues. Diets, for example, which are relatively high in calories but low in quantity and quality of protein will deplete the dispensable protein reserves of the animal, produce a fatty liver and fill the fat depots of the body. Rats, for example, fed 120 calories of a protein-free diet per kg. body wt. developed livers with an average of 1.1 gm. of protein and 57 per cent fat. Litter mates fed the same caloric intake but with 10 per cent protein developed livers with an average of 1.86 gm. of protein and 28 per cent fat. Increasing the calories in the absence of protein may indeed have little conserving effect upon body proteins, but it can have a marked conserving effect in the presence of protein. The total liver protein was increased from 1.27 to 2.30 gm. as the calories were increased from 50 to 200 gm./kg. body wt. in animals fed a constant protein intake.

Depletion of the protein stores of the body, either through lack of calories or lack of protein or both, results in an imbalance of tissue proteins and enzyme systems which can alter the physiology of the animal markedly. The plasma albumin, for example, is depleted rapidly, but plasma alpha globulins are not (31). The succinoxidase system of the tricarboxylic acid cycle is reduced in activity, while the cytochrome oxidase system is not (32). Thus, there can be a differential depletion and repletion of many types of protein systems depending upon the physiological state of the animal and the diet. The nitrogen balance method, however, does not describe these shifts between the various protein compartments of the body, but rather measures the overall status of nitrogen retention in the animal. Nitrogen balance is the sum of the gains and losses of nitrogen from the various compartments of the body. It is possible for an animal to be in positive nitrogen balance and yet be depleting some labile stores in protein. Nitrogen balance, like body weight or growth, is the summation of many variables and the significance of the balance becomes more meaningful as those variables are evaluated.

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FEDERATION PLACEMENT SERVICE

In the portion of the *History of the American Physiological Society*, written by Dr. Charles W. Greene and published in 1938 on the occasion of the semicentennial of the Society, it is recorded that as early as 1919 there was "ever-increasing discussion among Federation membership concerning the difficulties of young scientists ... in securing permanent positions" and reference is made to a proposed solution offered by Dr. Samuel F. Meltzer, a former President of the Society, to organize a "scientific information service" for which he contributed $300 of personal funds for the expense involved for the ensuing year. This proposal was then referred to the Federation, where it was adopted as a project; the Treasurer of the Physiological Society was made custodian of the funds and Dr. E. E. Brown of the University of Minnesota was appointed Director. As far as can be learned, Dr. Brown remained in charge of the Placement Service until sometime during the 1920's, when Dr. C. W. Edmonds assumed the duties. In 1931 Dr. H. B. Lewis of the Department of Biochemistry of the University of Michigan became the Director, giving generously of his time and interest to the project for over fifteen years. Small sums were made available each year by assessments on the constituent Societies to cover postage and stationery, and the use of a room at the Annual Federation Meeting was arranged in 1941. Dr. Lewis continued to serve until 1946, when he asked to be relieved but agreed to serve another year.

In 1947 a committee headed by Dr. Philip Dow made a study of the Placement Service and recommended that *1*) it be continued as a function of the Federation, *2*) it be operated by the Office of the Federation Secretary, and *3*) an annual fee of one dollar be paid by applicants, with the expectation that this would pay a secretary one-fourth time. This sum was soon inadequate to meet the expenses, for by 1948 they were already estimated at $1,300 and the Federation continued to provide the funds. When Dr. Milton O. Lee became Federation Secretary in 1947 the records of the Placement Service were moved to Washington to the new permanent office, and all subsequent activities have been under his direction.

From these available records it is evident that the original Placement Service of the Federation of American Societies for Experimental Biology was an outgrowth of the informal placement activity of every teacher of graduate students, to whom employers and colleagues frequently write seeking likely persons to fill available positions. For more senior positions those searching for the best available candidate may gather the opinions and recommendations from a number of individuals already leaders in that particular discipline. Such methods remain extremely satisfactory where they can function effectively, but since the field of experimental biology has become extensive, the employers of experimental biologists diverse and numerous, and the number of academic departments contributing to it so large, a central source for exchange of information under the auspices of the professional societies in that field has come to meet a real need that can no longer be effectively met by unorganized efforts.

In addition, it has been convenient for candidates for positions and employers seeking personnel to meet at the Annual Spring Meetings of the Federation. Hence the Placement Service has attempted to facilitate and expedite activities already occurring, by providing a central source of information for both employers and candidates to be available at all times, and a scheduled meeting place for them at the Annual Meetings, with information of the preparation, experience, and interests of each candidate present, in a brief and standardized form which is available to all employers.

During the last few years these activities have expanded to meet the increasing needs as they have become evident. Its main function has continued to be to serve as a clearing house of factual information for both candidates and employers, with no attempt to handle references or to recommend or evaluate candidates or positions. Originally, annual lists and monthly supplements of applicants with a brief description of their qualifications and experience were sent to all employers who registered positions or who wished to be kept on the mailing list, and interviews were arranged at the annual meetings by the Director, still on a rather informal basis with very few facilities. In 1950

at the Atlantic City Meeting a new system of scheduling interviews was used with a generous floor space allotted to the Service for offices and interview booths and a staff of about twelve assistants. This proved very satisfactory and was successfully repeated in 1951 at Cleveland with a few modifications and a slightly enlarged staff.

Meanwhile, as the number of applicants increased, the method of issuing the lists of available personnel was revised. In the summer of 1950 a questionnaire was sent to all members of the American Physiological Society asking the kind of information they wished in these lists and the intervals at which it was desirable to receive them. From the returns to this questionnaire it appeared that a schedule of quarterly lists of available personnel, semiannual lists of available positions, and an annual list of available fellowships was considered by the majority to be the most useful. Such a schedule has been adopted for this year with the quarterly lists of candidates prepared in February, May, August, and November, and the lists of available positions issued in March and September. The list of fellowships was issued in April of this year but probably would best be issued in the autumn on an annual basis. There have been many requests for these new lists of available positions and fellowships.

Detailed application forms of all applicants on the list are available for study upon request by an interested employer. In addition, there are on file the application forms of a few individuals, usually with some years of additional experience, who are available but do not wish their names circulated. Obviously as there are no facilities for work with individuals, the mechanism of the circularized lists is of no help in these instances, but the information so filed has been of considerable aid to the Director in making suggestions when he has been consulted in the filling of positions requiring this additional background. In the same way a number of positions are described for the files with the request that they not be included in the publicized list. These employers prefer to retain the initiative in approaching candidates. These procedures can be retained within the framework of the present activities, but it is to be emphasized that the Service can function most effectively where there is a free interchange of information.

During the past year an addressograph-plate mailing list has been assembled of heads of academic departments and laboratories hiring and/or training personnel in the fields of the member Societies and the circularized lists have routinely been sent to them, permitting the availability of both positions and personnel to reach a wide audience among potential candidates and employers even if they have not registered with the Service.

Such an activity becomes more expensive as its responsibilities increase and, while its value and standards can best be continued and developed under the direction of the Federation, it seems equitable that the cost should be shared by those who use it. As a step to accomplishing this, at the Cleveland Meeting in April 1951 the Executive Committee of the Federation set up a new schedule of rates, consisting of a three-dollar annual registration fee for candidates, and a ten-dollar subscription fee for all lists, with a five-dollar fee for any single copy. To be eligible to use the interview service at the Annual Spring Meeting an employer must be an annual subscriber to the lists or to the then current February list of applicants. It is estimated that this will provide an income to meet approximately half the cost of the Service, with the Federation supplying the remainder from its general funds. Thus the Federation will continue to direct the Service, which would be available to its membership and to non-members on much the same basis as in the past. From an over-all viewpoint the importance of this service to non-members should not be overlooked, for while it is recognized that the Service is most useful to young people just receiving their doctorate or possibly with a few years' experience, most of whom are not yet eligible for membership in the constituent Societies, the opportunity

TABLE 1. APPLICANTS AND POSITIONS REGISTERED, MARCH 1950–MARCH 1951

	APPLICANTS	% TOTAL	POSITIONS	% TOTAL
Physiology	173	38.3	177	34.8
Biochemistry	214	47.3	186	36.6
Pharmacology	38	8.4	85	16.7
Pathology	7	1.6	13	2.4
Nutrition	10	2.2	21	4.2
Immunology	2	.4	24	4.7
Miscellaneous	8	1.8	3	.6
Totals	452		509	

these candidates have for performing significant research in the following few years may well determine whether or not they will become useful members of the Federation. It follows then that the Federation may thus perform a very worthwhile function not only for the individuals concerned, but ultimately for the member Societies and for the fields of scientific endeavor they represent.

A review of the records of the Placement Service especially for the past year is of interest in indicating its present and possible future usefulness, and a summary of the registrations of the year ending March 1951 are presented in table 1.

TABLE 2. APPLICANTS AND POSITIONS REGISTERED AT CLEVELAND MEETING, APRIL 1951

	NO. APPLICANTS REGISTERED	NO. APPLICANTS INTERVIEWED	NO. INTERVIEWS	POSITIONS REGISTERED†
Physiology	52	41	160	86
Biochemistry	124	91	294	90
Pharmacology	18	16	62	35
Pathology	0	0	0	4
Nutrition	12	6	14	7
Immunology	3	2	2	4
Totals	209	156	532	226

In a period of expanding activity such as the present there are more positions than candidates to fill them, with accompanying competition in bidding for the services of well-prepared applicants and a consequent raising of all salary requirements and a careful selection on the part of candidates as to the opportunities presented by the position. This trend has become marked as more of the defense positions become available.

More pertinent and timely perhaps is table 2 showing the number of candidates registered at Cleveland divided into their fields of major interest. It must be pointed out that this division is arbitrary in some cases where candidates have more than one major interest or where the major interest is not included directly in the field of a member Society. In addition to the total registration, the number having interviews is recorded in *column 2*.

Of the 53 applicants not having interviews, about half did not register as present at Cleveland until the third day of registration, hence their application forms were not open to most employers when they first studied the forms. This points to a definite advantage to candidates of early attendance at the meetings.

It is interesting that a large number of biochemists, both candidates and employers, use the Service. This is not in proportion to the number of members of the Federation in that field, for while the registrations in biochemistry approximate half of the total in all six fields, the American Society of Biological Chemists has only about 70 per cent of the membership of the American Physiological Society and twice that of the American Society of Pharmacology and Experimental Therapeutics. Perhaps this is due in part to the general familiarity of all chemists with the very successful employment service of the American Chemical Society. The well-known scarcity of pharmacologists and immunologists is reflected very clearly in this table.

The positions were registered by a total of 161 employers present. Compared to the number of applicants registered, the relative number of positions does not seem to differ much from that in table 1, but a greater proportion of candidates than employers currently registered as active were present in Cleveland. Many non-member employers do not attend the Annual Meetings, while candidates seem to attend whether members or not.

To learn the qualifications of candidates which were most sought by employers at Cleveland, those receiving interviews were broken down by age groups and by degrees held. The following tables include only those for whom regularly scheduled interviews were arranged through the Placement Service.

These figures show the desire of employers for younger applicants, which is a well-recognized trend. The large number of interviews for the few applicants holding only a bachelor's degree shows a demand for trained technical assistants by these employers even though the applications of individuals not having advanced degrees have been discouraged, keeping to the policy that the Service should function chiefly on the professional level. It may also indicate that the present increase in all salary levels has forced some employers to lower their degree requirements if their funds are insufficient to meet the current range offered to candidates with advanced degrees.

Thus it would seem that the Placement Service

is meeting in a measure at least the needs of the professional group of employers as outlined above. These needs vary somewhat from the point of view of the employer and candidate.

The employer wishes objective information concerning the applicant's education and previous experience, with a chronologically complete record of his activities from the time he received his bachelor's degree, a brief account of investigative work participated in with the names of individuals under whom he may have worked, a bibliography

TABLE 3. APPLICANTS INTERVIEWED AT CLEVELAND
Breakdown by Age Groups

AGE GROUP	NO. APPLICANTS	NO. INTERVIEWS	INTERVIEWS/ PERSON
20–25	15	61	4.1
26–30	79	321	4.1
31–35	38	95	2.5
36–40	19	46	2.4
41–45	3	6	2.0
Over 45	2	3	1.5
Totals	156	532	

TABLE 4. APPLICANTS INTERVIEWED AT CLEVELAND
Breakdown by Degrees Held

DEGREE HELD	NO. APPLICANTS	NO. INTERVIEWS	INTERVIEWS/ PERSON
A.B. or B.S.	5	22	4.4
M.A. or M.S.	16	42	2.6
Ph.D., D.Sc., or M.D.	130	438	3.4
Ph.D. and M.D.	5	30	6.0
Totals	156	532	

of published work, a list of honors received and society memberships, and the salary he expects. The employer ordinarily prefers to obtain personal references directly and to evaluate the other information himself, for employers filling positions with experimental biologists are with few exceptions prepared by experience, association and knowledge of the field to do this best.

As mentioned before, the greatest number of candidates are recent graduates and are seeking an opportunity to continue experimental work, with or without teaching responsibilities, and at a salary adequate to meet the now constantly rising cost of living. Unless the degree was obtained under a department head who has few other administrative duties, many current contacts, few students and an agressive program for placing his students, the recent graduate may not know where to look for the position he has spent so long preparing for. Once the original contact is made the candidate may not feel able to evaluate the opportunities of the position. Since it is impossible for the Placement Service to assume this function it is necessary that he seek this assistance from his older and more experienced associates.

Not infrequently older, more experienced candidates, confronted by changed conditions or by changed interests, may wish to be considered for other positions. Unfortunately in the past there has been some stigma attached to the scientist having to seek employment. The world was expected to beat a path to his door. This is not a realistic attitude in these times and it is hoped that the short factual announcements as now prepared by the Placement Service of the Federation, which is interested only in maintaining and improving the standards within the profession, can remove the reasons for this attitude and the consequent reluctance by some to utilize its service.

When positions are plentiful as now, the Service is most useful to employers. In times of scarcity of positions its usefulness to candidates is increased. At all times the more widely this information is disseminated the more opportunity there is for an applicant to accept a post for which his training and interests prepare him. Such exchange of information also tends to equalize opportunities, conditions and salaries for positions of equal rank and responsibility, requiring similar training. The list of fellowships brings together in one place the essential facts about the fellowships available in the field of experimental biology, such as place and date of application, place and conditions of tenure, requirements and stipend for the consideration of both candidates and department heads when considering their own or their students' future plans.

Since it is desired to increase the usefulness of the Placement Service in accordance with the changing needs of the individuals using it, suggestions are welcomed.

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CORRECTIONS TO MARCH ISSUE

Page 167. BROQUIST, STOKSTAD AND JUKES, *title:* Delete word "crystalline."

Page 171 CILCOTE AND O'DEA. *Name of first author* should read, "MAX E. CHILCOTE."

Page. 177. DOUNCE, SIMMONS AND KAY, *line 3:* Change "sodium dodecyl sulfonate" to read "sodium dodecyl sulfate."

Page 189. GHOSH, WOODBURY, SAYERS AND SMITH, *line 12:* Change pH 8.0" to read "pH 1.0."

Page 227. NAJJAR, *line 25* (last line on page): Insert "μg." after "200".

Page 237. RIGGS, CHRISTENSEN AND RAY, *line 9:* Sentence should read: "When duck erythrocytes were suspended in saline medium containing elevated levels of glycine, glycine was taken up by the cells against the concentration gradient. . . ."

Page 400. ALTMAN AND EDSALL, *lines 14–15:* Instead of "Twenty-five of the remainder," read "Twenty-five per cent of the remainder."